OUTLINE OF THE
AMINO ACIDS AND PROTEINS

Edited by

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SECOND EDITION, REVISED AND ENLARGED

REINHOLD PUBLISHING CORPORATION
330 West Forty-Second Street, New York, U.S.A.
1948

Printed in the U.S.A. by
BERWICK & SMITH CO., NORWOOD, MASS.

Preface to First Edition

The purpose of this book is to outline in a simple and readable manner the essentials of the chemistry and the biochemistry of amino acids and proteins. We have attempted to present a clear and accurate picture of this difficult subject and have refrained, so far as possible, from becoming too deeply involved in the theoretical and argumentative aspects of the various hypotheses dealing with the chemical structure of proteins, of the relation of proteins to immunological reactions, of theories of denaturation and detoxication, as well as of certain thermodynamic considerations. Those who desire more detailed information on these phases of the problem should consult the larger treatises and the references cited.

An account of the discovery of the amino acids is briefly given. The story is interesting, stimulating, and revealing. Those investigators of the past who delved into the mysteries of the protein molecule and investigated the isolation and the chemical structure of the components of proteins have unearthed untold treasures. They freely gave us the benefit of their knowledge and experience. To the best of their ability, they described clearly the results of their investigations. Thus, they have laid open a wide and rich vista. This is our heritage. We must contribute to its future progress.

Every student of the biological sciences must have a basic knowledge of the chemistry and behavior of the proteins and amino acids. No diet is complete without proteins or their constituent amino acids. In fact, we can safely state that all living matter contains proteins and hence, in one sense, life is dependent on this particular form of nitrogen.

There is now great interest in proteins and amino acids. It is gaining momentum both in industry and in medicine. We must, therefore, present simply and concisely the published facts as we know them. It is possible that amino acids other than those listed and discussed in this book may commonly occur as constituents of protein molecules. Indeed, isolation of other amino acids from the hydrolyzates of particular proteins has been reported, and the presence of still others is suspected. Rigorous proof is lacking, however.

Those of us who are studying and investigating the chemistry of proteins and amino acids hope for better methods of isolation, for less costly and less complicated methods of synthesis, for more accurate methods of

determination, for a clearer concept of the metabolism and function of the amino acids, and for a better understanding of their nutritional significance. The elucidation of these and other aspects of the problem awaits further development. In war or in peace, science marches on.

MELVILLE SAHYUN

Detroit, Michigan
June, 1944

Preface to Second Edition

Since publication of the first edition of this volume considerable advances have been made in the field of proteins and amino acids, and much of the recent literature deals with the nutritional aspects of these substances. Hence the editor has undertaken the publication of a companion volume entitled PROTEINS AND AMINO ACIDS IN NUTRITION. However, among the newer findings has been the development of microbiological methods of assay of amino acids, which has indeed been a valuable contribution to this vast and complicated field. Therefore it was considered desirable to present herein a brief review of the literature that led to this achievement and to give an outline of the most reliable and wide-spread methods used, hoping that the reader would find this chapter informative and useful. Any omission of names of investigators or results of investigations is inadvertent, and in advance I beg for indulgence and offer my sincere apologies.

THE EDITOR

Detroit, Michigan
October, 1947

Acknowledgement

The writer is greatly indebted to Professor Carl L. A. Schmidt and to Professor D. M. Greenberg of the Department of Biochemistry of the University of California, for valuable suggestions and criticisms and for their assistance in editing the manuscripts of this book, and to Mrs. Harriet Wood for her painstaking work in making original drawings of the amino acid crystals that were prepared and examined microscopically. Copies of the photographs of some of the early scientists who greatly contributed to our knowledge of proteins and amino acids were obtained from Professor Schmidt's collection. Indeed, I cannot adequately express to Professor Schmidt my appreciation and thanks for his constant encouragement, enthusiasm, and unselfish spirit that enabled me to undertake and complete this work.

MELVILLE SAHYUN

Foreword

Although Wollaston, in 1810, isolated cystine from a urinary calculus and Proust in 1819 obtained impure leucine from fermented wheat gluten, it was not until 1820 that Braconnot obtained glycine from the acid hydrolyzate of gelatin. This marks the beginning of modern protein chemistry. With the exception of cysteine and the questionable beta-hydroxyglutamic acid, a total of 21 amino acids have been isolated from proteins and their chemical structures proved by syntheses. The occurrence in proteins of several other amino acids has been reported, but the evidence is as yet not sufficiently conclusive to warrant general acceptance. There is reason to believe that amino acids other than those that are now considered as accepted may occur in some proteins. However, clear-cut demonstrations have not yet been made. It is important that this subject be extensively explored.

It is now quite firmly established that the amino acids constitute the building stones of proteins. A prerequisite to the elucidation of the structure of proteins is a knowledge of their content of amino acids. In most cases complete data are lacking. Systematic studies along these lines should be made. A handicap to such work is shortcomings in some of the methods for estimating the amino-acid content of proteins. Some of the reported data are not reliable, because of impurity and non-homomolecularity of the preparations subjected to analysis. There is still no generally accepted view as to the details of protein structure. The peptide bond as the mode of linkage between the amino-acid residues seems to be firmly established. Ultimately it is necessary that protein molecules be represented by structural formulas. At present, this time appears to be very distant.

It should not be inferred from the above that tremendous advances have not been made in our knowledge of the chemistry and behavior of proteins. The now classical experiments of Hardy on the influence of pH on the path of migration of protein molecules when subjected to the action of a direct current marks one of the early milestones in the application of physical chemistry to this subject. The amphoteric nature and the zwitterionic structure of amino acids and proteins are now definitely proved and accepted. The seat of acid- and base-combining capacity of proteins has been elucidated, although from a stoichiometric standpoint further work needs to be done to make the data quantitative. The molecular weights of many of the proteins in various solvents have been determined with a fair degree of certainty. These data have shed important light on

the state of aggregation or micellation of some proteins. Thermodynamic data, especially those relating to amino acids, have done much toward establishing a firm foundation upon which to build our concepts of the behavior of these compounds both in the solid state and when present in solution. X-ray studies of proteins have led to important deductions regarding the stretching of protein fibers as well as supplying information on the basis of which deductions as to protein structure can be made. Electrophoresis has proved a powerful tool in showing that many of the proteins that at one time were regarded as chemical entities are really mixtures of one or more closely related proteins. In other cases this technique has added further evidence of homomolecularity. It is probably not too bold to state that the physical chemical and thermodynamic characterization of amino acids and proteins has eclipsed all other phases of protein chemistry, especially the organic, although in this connection the new methods that have been developed for synthesizing peptides should not be minimized.

Crystallization of many of the enzymes has been achieved. The present evidence indicates that enzymes are proteins or contain a protein as a component. Much has been done to explain the mode of action of enzymes, especially those containing prosthetic groups (coenzymes). However, there is still no adequate explanation of the specificity of enzymic action, particularly of those that participate in hydrolytic reactions, or even a proper concept as to how such enzymes function as catalysts. For example, it is not known why pepsin cannot hydrolyze proteins to amino acids. Data on this subject are accumulating slowly, but much work remains to be carried out before the fundamental questions can be answered satisfactorily. The overall action of those hormones that are proteins is known, but knowledge regarding the chemical mechanism whereby these compounds produce their effects is lacking. A most fascinating field of endeavor has concerned itself with the chemistry of the filtrable viruses. The fact that these substances are nucleoproteins and that they can, when placed on a suitable substrate, reproduce their kind affords a new viewpoint as to the importance of protein in life processes. It is not altogether wishful thinking to hope that eventually information regarding the genes may be brought to light.

Important advances have been made to our knowledge of the nutritional requirements of amino acids by different animals. This has paved the way for studies dealing with differences in metabolism of various species. Comparative biochemistry offers a rich field for future studies. With the discovery and availability of isotopes, both radioactive and non-radioactive, a most powerful tool for metabolic studies has been provided. In many instances it is now possible to tag molecules and thus follow the fate of the constituent atoms in the body. Studies on isotopic amino acids have shown that proteins are extremely labile structures. The components of proteins are continually disappearing, reappearing, and changing into one

another. The terms endogenous and exogenous metabolism no longer have the significance that they once did.

The relation of proteins to immunity has long been known. The specificity of immunological reactions depends on the chemical makeup of the components that participate. The immune bodies are protein in nature. There are indications that important advances relating to the chemistry of immune bodies may be expected in the not too distant future. It is no idle wish to hope that the day may come, as it already has in the case of many of the vitamins, that we may not altogether have to depend upon biological sources for our supplies of such important and indispensable products as the enzymes, hormones, and immune bodies, but that it will be possible to synthesize them in the laboratory.

Not a few of the present-day workers remember the time when many of the amino acids were a rarity. Others were still unknown. The state of purity was often questionable. Much of this picture is now of historic interest. The large-scale production for flavoring purposes of monosodium glutamate from wheat gluten in the Orient was the first instance of the industrial use of an amino acid. This product is now being obtained in large quantities in the United States from Steffen's sugar waste. The medical profession has come to recognize that intravenous administration of amino acids has a logical place in certain types of therapy. Large-scale production of amino-acid solutions has supplied this demand. It is altogether likely that specific uses will be found for many of the amino acids. All this will make it possible for the laboratory worker to study with greater ease many more of the interesting properties of these compounds. The day has almost passed when it was necessary to go through the tedious and laborious procedure of isolating from a protein the particular amino acid upon which studies were to be carried out.

In recent years several comprehensive texts dealing with the chemistry of the amino acids, peptides, and proteins have appeared. They have been written primarily for the use of individuals who possess an adequate background for understanding the subject. An elementary text that will serve as an introduction to this field is lacking. The present book is an attempt to fill this gap, at least in part. It does not aim to cover all phases of the subject nor does it attempt to treat any subject exhaustively. On the basis of such information as the text does supply, the reader should find it easier to widen his knowledge of amino acids and proteins by subsequently studying the larger texts and eventually, as he should always do, by consulting the original articles to which reference has been made.

CARL L. A. SCHMIDT

Contents

Chapter I

Discovery of the Amino Acids

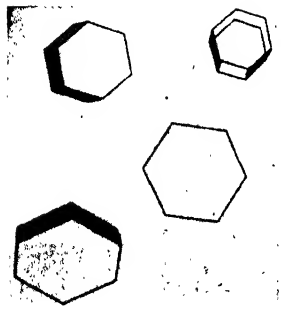
MELVILLE SAHYUN

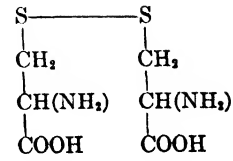
The Research Laboratories, Frederick Stearns & Company, Detroit, Michigan

Louis Nicolas Vauquelin

Born in Saint-André, Normandy, in 1763; died in 1829; discovered chromium and confirmed the discovery of lithium. Also the discoverer of cyanic acid, benzoic acid in the urine of herbivora and acetic acid in wood distillate; with Robiquet he isolated asparagine. His work had a great influence on the entire field of chemistry.

CYSTINE





Cystine was the first amino acid to be discovered, but ninety-five long years of research were necessary to determine definitely its origin and con-

stitution. Probably no other amino acid has attracted so much attention and created so much interest.

The discovery of cystine was not related to a search for its presence in protein hydrolyzates. In 1810, Wollaston [162] isolated a crystalline substance from a urinary calculus and termed it "cystic oxide." He obtained it as hexagonal plates by dissolving the calculus in an alkali and acidifying the solution with acetic acid. Although he burned a sample and noted the unpleasant characteristic odor, he failed to identify the presence of sulfur. In 1832, Berzelius [11] acknowledged Wollaston's discovery, but pointed out that the term "cystic oxide" was not appropriate for this organic compound. He changed the name to cystine.

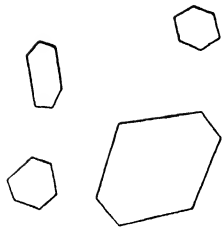
In 1837, Baudrimont and Malaguti [7] showed that cystine contained sulfur. Liebig [96] became interested in this subject and confirmed their findings. Years of intensive research followed the earlier investigations on cystine, and yet this elusive amino acid was not related to the acid hydrolyzates of protein. As a matter of interest, it may be pointed out that up to 1873 sulfuric had been the acid of choice for the hydrolysis of proteins. Sulfuric acid was then removed as its calcium salt and the insoluble calcium sulfate was discarded. It was, therefore, probable that cystine was lost in the precipitate. Later when hydrochloric acid was employed, investigators resorted to the addition of stannous chloride in order to reduce the formation of humin during the acid hydrolysis of proteins. In the light of present knowledge, one can readily see how cystine eluded investigators by this procedure, as it was reduced in large part to the more soluble amino acid, cysteine.

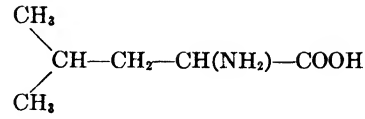
It was not until 1899 that Mörner [100] reported before the Swedish Academy his findings of cystine in the acid hydrolyzate of horn. Almost simultaneously, Embden,[38] ignorant of Mörner's discovery, hydrolyzed horn with concentrated hydrochloric acid and isolated cystine.

The identity and constitution of this simple, elusive compound were by no means settled. The position of the thiol group in relation to the amino group in the molecule was a subject of controversy. At the time of Mörner's discovery, both the amino and the thiol groups were believed to be linked to the carbon atom in the alpha position. The brilliant investigations of Neuberg [106] and of Friedmann [62] helped considerably to clarify the situation and establish the position of the amino group in the alpha position and that of the thiol group in the beta position. In 1905, the chemical formula of cystine was well established and its accuracy was confirmed by Erlenmeyer, Jr.,[44] who synthesized cystine from benzoyl serine.

However, the old controversy persisted as to whether or not the cystine obtained from protein hydrolyzates was identical with that cystine present in urinary calculi. Fischer and Suzuki [58] settled the argument by demonstrating that the points of difference between protein and urinary cystine were due to the presence of tyrosine in the latter.

LEUCINE





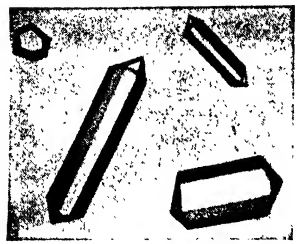
Leucine in its purest form consists of beautiful, very thin, white lustrous crystals. In 1819, Proust [124] discovered it accidentally while carrying out some fermentation experiments in connection with the flavor of different types of cheese. He first investigated gluten; later, he used milk curds and obtained from both proteins a white crystalline substance which he termed "acide caseique." The leucine of Proust, as well as that of most investigators of the nineteenth century, was a white powdery substance, and microscopically consisted of nodules or balls of needles (probable indication of tyrosine contamination). It is doubtful that Proust obtained a pure product. Almost a year after he had announced his discovery, Braconnot [16] obtained leucine from the acid hydrolyzates of muscle and wool, and it was he who gave it the name leucine. Like most other investigators in those early years of pioneering in the field of protein chemistry, Braconnot had neither accurate methods of analysis nor adequate laboratory facilities.

Mulder,[103] who originated the term *protein*, became interested in leucine. In 1839, he obtained it from the alkaline hydrolyzates of muscle and wool. He went a little further in his analysis than Braconnot had done, and noted its solubility in water and recorded its melting point. Mulder's leucine was considerably purer than that of his contemporaries, and it is believed that he alone of the early investigators had isolated it in almost pure form. In 1902, Habermann and Ehrenfeld [65] described a method of separating leucine from tyrosine, the basis of which was a difference in the respective solubilities of the two amino acids in glacial acetic acid.

The first correct formula for leucine, $C_6H_{13}NO_2$, was published in 1848 by Laurent and Gerhardt.[89] However, the structure of this amino acid as well as of the other four that had then been isolated, was still unknown. Several years of research followed the early investigations on this amino acid, but it was not until 1891 that the structural formula of leucine was established by Schulze and Likiernik [138] and natural leucine was shown to be identical with the synthetic product.

Synthesis of leucine was accomplished by Erlenmeyer, Jr., and Kunlin,[46] Fischer and Schmitz,[56] and Bouveault and Locquin.[14] Fischer and Warburg [59] resolved the racemic leucine into its components.

Glycine



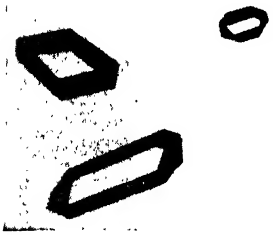
$CH_2(NH_2)—COOH$

During the early part of the nineteenth century, the problem of sugar production from vegetable matters other than cane-sugar reigned supreme among scientists of continental Europe. It was only natural that eminent chemists like Braconnot [16] should have attempted to find out if sugar could be produced, not only from vegetables, but from animal sources. Previous to his discovery of glycine, Braconnot had demonstrated that sugar could be obtained from the acid hydrolyzates of wood, bark, straw and hemp. In 1820, he investigated the degradation products of animal matter. He boiled gelatin in sulfuric acid for five hours and neutralized his acid hydrolyzate with calcium carbonate. He then removed the insoluble calcium sulfate, concentrated the filtrate to a sirup and permitted it to stand. A month later, there were beautiful white crystals adhering to the walls of the container. Braconnot recovered the sweet-tasting crystals which he termed "sucre de gélatine" — gelatin sugar. To the best of our knowledge, this was indeed the first instance in which a pure amino acid was obtained from the acid hydrolyzate of proteins. Unfortunately, Braconnot was not a very thorough investigator, for other than observing some of the physical characteristics of this amino acid and noting that it was more easily crystallized than cane sugar, he made no further chemical studies. He did not even observe that it contained nitrogen.

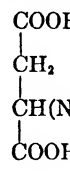
In 1838 Mulder,[102] the energetic chemist, became interested in this amino acid and showed that gelatin sugar and leucine could be obtained by the hydrolysis of gelatin with potassium hydroxide. His early studies on the chemistry of this simple amino acid aroused a good deal of sharp criticism from French scientists. In 1845, Dessaignes [28] successfully isolated glycine from the cleavage products of hippuric acid and gave it an accurate formula, but admitted that he did not have conclusive evidence.

In 1846, Horsford [74] from Liebig's laboratory, Laurent,[88] and Mulder [102] reported accurately on the chemical composition of this amino acid. Probably at the instigation of Liebig, Horsford applied the term "glycocoll" in place of "sucre de gélatine." Two years later, Berzelius, one of the most eminent organic chemists of his day, named it glycine.

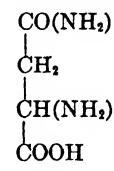
In 1857, Cahours [17, 18] elucidated the structural formula of glycine and in 1858 he published his synthesis, the principle of which consisted of treating monochloroacetic acid with ammonia. A year later Perkin and Duppa [118] confirmed Cahours' findings and prepared glycine by treating monobromoacetic acid with ammonia.



ASPARTIC ACID



ASPARAGINE



In 1806, two French chemists, Vauquelin and Robiquet,[149] isolated a crystalline substance, which they termed asparagine, from the juice of asparagus. They were unable to determine its chemical composition. Later, they observed that these crystals were of two types. In their meager report, it was stated that the crystalline substance yielded no ash and probably consisted of carbon, hydrogen, oxygen, and possibly nitrogen.

In 1827, Plisson [121] repeated an experiment which Bacon [3] had performed the year before on the root of the marshmallow, and from which he had obtained a crystalline substance, which in his opinion was a salt of malic acid. Plisson reported that Bacon's compound was neither a salt nor an acid but a compound having the properties of asparagine. He treated an aqueous solution of this substance with lead acetate and removed the lead with hydrogen sulfide. From the mother liquor he obtained white, shining crystals that were but slightly soluble in cold water, but were very soluble in hot water. To this crystalline substance, Plisson gave the name of aspartic acid.

In 1833, Pelouze [117] investigated the chemical nature of aspartic acid and communicated his findings to Liebig,[94] who immediately became interested in the problem. Liebig spent several years studying the chemistry of the conversion of asparagine to aspartic acid. In 1838, he hydrolyzed asparagine with potassium hydroxide, crystallized the aspartic acid so formed and reported an accurate empirical formula.

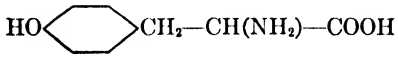
The elucidation of the structural formula of aspartic acid was conducted by Piria [119] at the University of Pisa. His results were not very conclusive, but as an outcome of his investigations he brought to light some very valuable findings. He showed that when asparagine was boiled with hydrochloric or with free nitric acid it was converted to aspartic acid. Furthermore, both asparagine and aspartic acid lost their nitrogen (deaminized) when treated with nitrous acid. This indeed was the first mention in the literature of the effect of nitrous acid on deamination.

Following Piria's publication, Dessaignes [28] succeeded in synthesizing aspartic acid. However, neither his nor Engel's synthesis helped to elucidate its chemical structure. This was accomplished by Piutti [120] in 1887.

Aspartic acid was not associated with the products of acid hydrolysis of proteins until 1868, when Ritthausen [127] succeeded in isolating it along with glutamic acid. His procedure was based on Scheele's method. (In 1785, Scheele had applied it to the isolation of malic acid.) After separating the insoluble tyrosine and leucine from the acid hydrolyzate of proteins, Ritthausen allowed the mother liquor to stand over sulfuric acid, whereupon a considerable amount of glutamic acid separated. After the isolation of glutamic acid crystals, the clear filtrate was observed to be strongly acidic. He neutralized the mother liquor by the addition of barium carbonate, filtered off the excess of barium carbonate, and added alcohol to the liquid. On standing, the barium salt of aspartic acid crystallized. Unfortunately, he was misled by his own analysis as to the nature and identity of his compound. This did not discourage him in the least. In 1869, he published the results of further investigations of this substance. This time, he used calcium oxide instead of barium carbonate and separated the calcium salts of the carboxylic amino acids by the addition of alcohol. He found that he had obtained a mixture of two crystalline products. By the addition of about 60 per cent alcohol he was able to remove the calcium salt of aspartic acid. The calcium salt of glutamic acid being more soluble in this concentration of alcohol remained in solution. He further observed that the copper salts were extremely useful in separating small quantities of aspartic acid.

Ritthausen showed that aspartic acid is a constituent of vegetable proteins. Kreusler [86] reported that it is also present in casein and in egg proteins. It is now recognized that aspartic acid is widely distributed in proteins.

Tyrosine



In 1846, Liebig [95] discovered tyrosine during the course of his investigation of the decomposition of proteins with alkali. He fused crude casein with an equal weight of potassium hydroxide, dissolved the mixture in hot

water and acidified it with acetic acid. On cooling, a white crystalline substance separated which he termed tyrosine. Although his preliminary analysis was faulty, he pointed out its amphoteric properties. Liebig made no attempt to determine its structure. Two years later, de la Rue,[27] who was then studying the composition of the cochineal insect, isolated tyrosine in the pure state and determined its empirical formula. About that time, Liebig, who had several other investigations under way, turned the problem over to Bopp.[13] In 1849, Bopp investigated the conditions under which tyrosine could best be obtained from various proteins. The original fusion method was not a satisfactory procedure for a good yield of this amino acid. He noted that tyrosine and leucine were not destroyed by prolonged boiling in hydrochloric or in dilute sulfuric acid.

About the middle of the past century there was considerable controversy on the question of acid hydrolysis. Braconnot had shown that leucine could be obtained from the acid hydrolyzates of proteins. Mulder had contended that the use of hydrochloric acid was not satisfactory, as it led to the formation of ammonia and ammonium salts of humic acid. Bopp reinvestigated the acid hydrolysis of proteins. He treated casein with hot hydrochloric acid and carefully noted the various changes in color formation that took place. After boiling the mixture for about eight hours, he obtained both tyrosine and leucine from the acid hydrolyzate. Bopp's experiment is of considerable importance as it is the first one on record relating to the hydrolysis of proteins by hydrochloric acid.

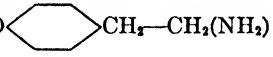
Bopp's method of separating tyrosine from leucine by fractional crystallization from dilute alcohol was not satisfactory. In 1902, Habermann and Ehrenfeld [65] showed that glacial acetic acid dissolves leucine but not tyrosine.

Tyrosine is widely distributed in nature. Frerichs and Städeler [61] were the first to demonstrate the presence of tyrosine and leucine in diseased liver. Later, they showed it to be present in blood and in urine. In 1861, Bodeker [12] reported on the relation of tyrosine to homogentisic acid, a substance associated with alcaptonuria.

In 1865, Schmitt and Nasse [133] undertook the problem of the elucidation of the chemical structure of tyrosine and concluded that it was related to salicylic acid for the following reasons: (1) on the basis of Städeler's findings, tyrosine could be converted to chloranil; (2) on dry distillation, it yielded phenylalcohol; (3) it gave a colored compound when treated with ferric chloride; and (4) it was a dibasic acid. They were unable to synthesize it.

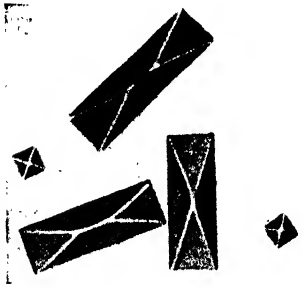
On alkaline fusion of tyrosine, Barth [6] obtained acetic acid and parahydroxybenzoic acid. On the basis of this evidence, he disproved the assumption of Schmitt and Nasse that tyrosine was related to salicylic acid. Ost [114] confirmed the findings of Barth, but failed in his attempt to synthesize tyrosine.

The synthesis of tyrosine was first accomplished by Erlenmeyer and Lipp.[41] They treated para-amino-phenylalanine with nitrous acid. Erlenmeyer, Jr. and Halsey [45] prepared it by condensing hippuric acid with para-hydroxy-benzaldehyde in the presence of acetic anhydride and sodium acetate to form the lactimid (azlactone). On alkaline hydrolysis of the lactimid and subsequent reduction with sodium amalgam, benzoyl-tyrosine separated and was converted to tyrosine by acid hydrolysis. In 1911, Wheeler and Hoffman [155] reported an interesting synthesis consisting of boiling anisalhydantoin with hydriodic acid and red phosphorus. In this reaction, reduction of the double bond takes place, ammonium iodide is liberated, the hydantoin ring is opened and the urea grouping undergoes hydrolysis, all in one operation. Emil Fischer [47] was the first to accomplish the separation of the optical isomers of the synthetic tyrosine.

TYRAMINE 

Tyramine, a substance of considerable physiological interest, is formed by the decarboxylation of tyrosine. The chemical reaction was first accomplished by Johnson and Daschavsky,[78] who treated tyrosine with a mixture of diphenylmethane and diphenylamine. Abderhalden and Gebelein [1] succeeded in obtaining tyramine by heating tyrosine with diphenylamine.

ALANINE



$CH_3—CH(NH_2)—COOH$

Alanine is the first amino acid that was obtained by synthesis before it was shown to be a product of the hydrolysis of proteins. In 1850, Strecker [147] investigated the synthesis of lactic acid and thought that it should be possible to make it from aldehyde and formic acid. He reasoned: since on oxidation, mandelic acid yields benzaldehyde, and since mandelic acid may be prepared by treating benzaldehyde with a mixture of hydrocyanic and hydrochloric acid, then the reaction involves the action of nascent formic acid on benzaldehyde. Thereupon, Strecker treated aldehyde ammonia with a mixture of hydrocyanic and hydrochloric acid. On concentrating the solution, ammonium chloride separated. The excess of hydrochloric acid was removed as lead chloride and that of lead by hydrogen sulfide. On heating the mother liquor and concentrating it, a crystalline

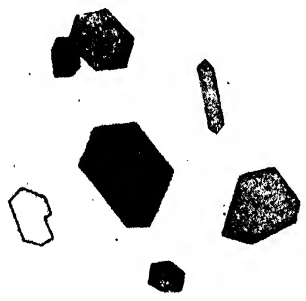
substance appeared which was not lactic acid. He called it alanine (alpha-amino-propionic acid). He later obtained lactic acid by treating alanine with nitrous acid.

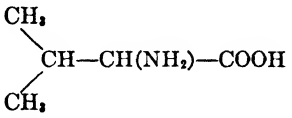
The discovery of alanine among the products of protein hydrolysis is claimed by two separate investigators. In 1875, Schützenberger and Bourgeois [142] isolated a crystalline substance from an alkaline hydrolyzate of silk, which resembled Strecker's alanine. They termed it "leucine propionique." Their analysis was in agreement with the theoretical composition of alanine; but unfortunately they made no other tests nor prepared any other characteristic salts to justify their claims fully. In fact they based their claim on one single experiment.

In 1888, Weyl [154] studied the acid hydrolyzate of silk. After repeated crystallization, he obtained large rhombic crystals which upon analysis gave results that were very close to the theoretical composition of alanine as given by Strecker. He also prepared the copper salt of his compound and proved its chemical composition. In his paper, Weyl stated that he could find no leucine among the products of silk protein, contrary to some of the findings of earlier investigators. His statement was not far from the truth, for according to the best analytical methods, there is only approximately two per cent leucine in silk fibroin.

It was only after Fischer's work that the wide distribution of alanine was appreciated.

VALINE





In 1856, von Gorup-Besanez [153] announced his discovery of valine. He prepared aqueous extracts of liver, spleen, thymus, and pancreas, and heated the extracts in order to coagulate the proteins. His main interest at that time was a study of the leucine and tyrosine contents of glands. He found a substance in the pancreas that greatly resembled leucine in its behavior but differed from it in being more insoluble in boiling alcohol. He crystallized it, determined its chemical composition, and classified it as one of the homologous series of compounds which included glycine, alanine and leucine.

Almost a quarter of a century passed before valine was obtained as a constituent of protein hydrolyzates. In 1879, Schützenberger [141] demon-

strated its presence among the cleavage products in the hydrolyzate of albumin. At that time it was called aminovaleric acid. Four years later, Schulze and Barbieri [135] prepared it as its copper salt from extracts of the sprouts of *Lupinus luteus.*

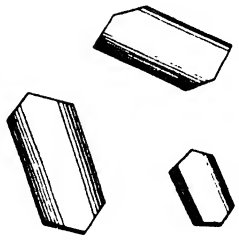
In 1866, Clark and Fittig [22] investigated its chemical structure. The empirical formula of valine offered several possibilities: amino-*n*-valeric acid, aminoisovaleric acid or ethyl-methyl-aminoacetic acid. Clark and Fittig claimed the synthesis of alpha-aminovaleric acid. Their synthetic preparation resembled the natural product of von Gorup-Besanez in its solubility, but differed from it in its melting point. This was attributed to impurities present in the natural amino acid. von Gorup-Besanez accepted Clark and Fittig's conclusions that their synthetic valine was identical with the natural product.

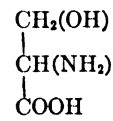
Other investigators were not convinced of the accuracy of Clark and Fittig's conclusions. In 1878, Schmidt and Sachtleben [132] synthesized alpha-aminoisovaleric acid and showed that their preparation was identical with that of Clark and Fittig. Consequently, the latter could not have synthesized alpha-aminovaleric acid. Lipp [97] confirmed Schmidt and Sachtleben's work. Thus the constitution of valine was once more in a state of confusion. Unfortunately, the relation of the optical activity to the properties of valine was not taken into consideration.

The elucidation of the structural formula of valine was finally accomplished by Emil Fischer.[49] He separated the *d*- and *l*(+)- forms from the racemic alpha-aminoisovaleric acid and showed that the *d*-valine was identical with that isolated from *Lupinus luteus* by Schulze. He also observed that the *d*(−)-valine was decidedly sweet in taste whereas the *l*(+)-valine was both bitter and sweet.

About 1901, Fischer [49] introduced the method of esterification of amino acids and separation of the esters by fractional distillation at reduced pressure. From the fraction that distilled between 40°–80° C at 10 mm, he isolated α-aminoisovaleric acid and named it valine.

Serine





In 1865, Cramer [23] isolated the gelatin-like protein found on the surface of the fibroin of silk and named it sericine. He prepared a sample of about 6 grams and hydrolyzed it with sulfuric acid. After the removal of tyrosine

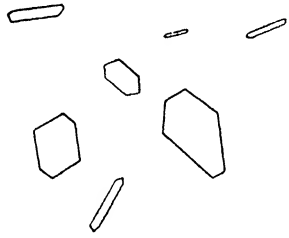
from the hydrolyzate, he obtained a crystalline substance that he at first considered to be glycine. When Cramer prepared its copper salt and noted its low copper content, he carefully reinvestigated the problem and concluded that he was dealing with a new amino acid, the chemical composition of which differed from that of alanine by one oxygen equivalent. He termed it serine and prepared the hydrochloride, nitrate, and sulfate salts.

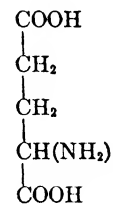
For about 30 years Cramer's amino acid was not referred to in the literature. It is a difficult one to isolate and, comparatively speaking, it is a rare amino acid. Fischer and Skita [57] prepared serine from the amino acid esters of high boiling point derived from the hydrolysis of silk. Owing to the drastic treatment to which the hydrolyzate was subjected during the process of esterification, their serine was obtained in the racemic form. However, they confirmed Cramer's discovery. Kossel and Dakin [84] obtained serine from the protamines and later from other proteins.

In 1902, Fischer and Leuchs [53] synthesized serine from glycolic aldehyde by the cyanohydrin method of Strecker. Other methods of synthesis were reported by Erlenmeyer, Jr.[43] and by Leuchs and Geiger.[93] Fischer and Jacobs [52] resolved racemic serine into its optical isomers by a chemical method. Ehrlich [34] performed the resolution by means of yeast.

Serine has been reported to be present in human sweat by Embden.[38] It was isolated from alfalfa leaves by Vickery.[150] According to Daft and Coghill [25] strong alkalies decompose serine.

GLUTAMIC ACID





Glutamic acid is of considerable interest since it is the only amino acid that has found extensive commercial use. The monosodium salt is used as a condiment.

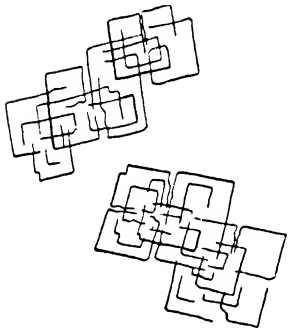
In 1866, Ritthausen [128] investigated the amino acid content of wheat gluten by subjecting it to sulfuric acid hydrolysis. After removal of sulfuric acid with calcium hydroxide, he observed that the clear filtrate contained a strong acid capable of decomposing calcium carbonate. Thereupon, he treated the filtrate with calcium carbonate, precipitated the excess with oxalic acid, and the excess of oxalic acid with lead carbonate. Following the removal of the excess of lead by means of hydrogen sulfide, and concentration of the filtrate, a considerable mass of crystalline matter was deposited. In this crystalline mass he identified tyrosine. After the

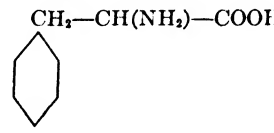
removal of tyrosine, he isolated gleaming rhombic crystals which he later identified as glutamic acid. Ritthausen repeated his experiments by substituting gliadin for gluten and obtained a 30 per cent yield of this new compound. This enabled him to prepare its barium, copper and silver salts for the purpose of identification and establishing the empirical formula.

In 1873, Hlasiwetz and Habermann [72] introduced a novel method for the hydrolysis of proteins. They used hydrochloric acid in the presence of stannous chloride and observed that the addition of the latter prevented the formation of humin. They hydrolyzed casein according to the above method and noted that the hydrolyzate contained a large amount of glutamic acid in addition to leucine, tyrosine, aspartic acid and ammonia.

The synthesis of glutamic acid was first accomplished by Wolff.[161] He employed a novel method using levulinic acid as his starting material.

PHENYLALANINE





Posen [122] on one hand, and Schulze and Barbieri [135] on the other, claimed the discovery of this essential amino acid in 1879. Posen's claim was based on his alleged synthesis of phenylaminopropionic acid by treating bromohydrocinnamic acid with ammonia, but his identification of the product was not accurate. He reported that it was soluble in alcohol and had a melting point of about 120°. These properties resemble those of benzoic acid more than of phenylalanine.

Schulze and Barbieri [135] almost simultaneously announced their discovery of phenylalanine. They isolated it from the sprouts of lupine, prepared its copper salt and accurately reported its empirical formula.

Following the report of Schulze and Barbieri, Erlenmeyer and Lipp [41] announced the synthesis of phenylaminopropionic acid and adopted the name phenylalanine. Despite the fact that phenylalanine is widely distributed in nature, it is a difficult amino acid to isolate.

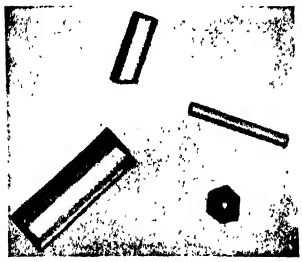
Numerous investigators have reported on the synthesis of phenylalanine:

Erlenmeyer, Jr.,[41] Knoop and Hoessli,[81] Fischer,[51] Posner,[123] Sörensen,[145] Wheeler and Hoffman,[155] Johnson and O'Brien,[79] Sasaki,[131] etc.

The resolution of the racemic phenylalanine into its optically active isomers was accomplished by Fischer and Mouneyrat.[55]

LYSINE

$NH_2—CH_2—CH_2—CH_2—CH_2—CH(NH_2)—COOH$



Lysine HCl

Lysine Picrate

In 1889, Drechsel[32] became interested in Schützenberger's observation relating to the evolution of carbon dioxide, when proteins were digested with an alkali. First, he hydrolyzed casein by Hlasiwetz and Habermann's method. He observed that little, if any, carbon dioxide was formed during hydrolysis. He then removed the tin, concentrated his hydrolyzate to a sirup and separated the crystallizable fractions. To the sirupy mother liquor he added phosphotungstic acid, a reagent long known to be an alkaloid precipitant. The heavy precipitate that formed was filtered, washed and decomposed with barium hydroxide. The filtrate was acidified with hydrochloric acid and concentrated. On standing, a crystalline substance was deposited. It was recovered, and recrystallized from a mixture of water and alcohol. Drechsel observed the strong basic properties of the new amino acid. He prepared the chloroplatinate and silver salts, and noted that this amino acid was quite stable in the presence of strong acids; but on treatment with barium hydroxide, it decomposed with the liberation of barium carbonate. He also isolated urea from the decomposition products. Drechsel named the new compound *lysatine* (later shown to be a mixture of lysine and arginine).

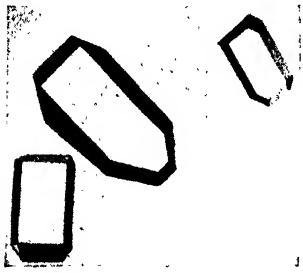
Drechsel repeated his experiments on the phosphotungstic acid fraction of protein digests. He obtained some perplexing results, particularly when he attempted the isolation of the silver salt of his lysatine.

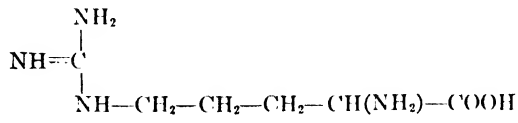
The isolation and identification of lysine in a pure state were accomplished in Drechsel's laboratory by his three assistants, Siegfried, Ernst Fischer and S. G. Hedin.[30] Ernst Fischer termed it lysine. Hedin isolated it from the pancreatic digest of fibrin and also prepared lysine chloro-

platinate in accordance with Drechsel's pattern. None of these investigators was successful in crystallizing lysine as the free base. This was accomplished by Vickery and Leavenworth [152] in 1928.

The elucidation of the chemical structure of lysine was finally accomplished by Fischer and Weigert,[60] who synthesized it from the cyanopropylmalonic ester by treatment with nitrous acid and subsequent reduction.

ARGININE





In 1886, Schulze and Steiger [139] discovered arginine in the aqueous extracts of etiolated lupine seedlings. They observed that the addition of phosphotungstic acid to the aqueous extracts gave a copious, white precipitate. From this precipitate they separated a new crystalline compound which possesses basic properties and is precipitable at neutral or alkaline reactions by mercury salts in the presence of sodium carbonate. Their first report was followed by another. They showed that arginine was stable when heated with strong acids and decomposed when heated with alkalies giving rise to carbon dioxide and ammonia. Nitrous acid removed only one-fourth of its nitrogen. It contained neither sulfur nor phosphorus. It was precipitable by most of the known alkaloid reagents.

Schulze and Steiger also investigated the amount of arginine produced during the sprouting of lupine seeds and concluded that proteins must have been converted (in part at least) to arginine during the process of germination. They also demonstrated that arginine was decomposed by alkali with the production of urea. This latter observation led Schulze and Likiernik [138] to associate Drechsel's discovery of lysine and lysatine with their new compound and to its possible presence among the products of protein digestion.

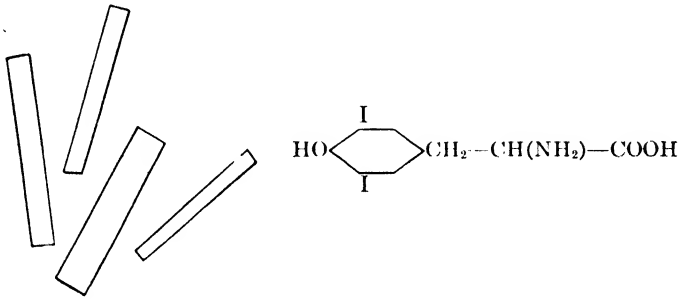
Drechsel and his group,[30] who had been diligently working on lysine and lysatine, became interested in Schulze's arginine. However, it was Hedin who finally isolated the silver salt of arginine from the phosphotungstic acid precipitates of protein digests. He reinvestigated the whole problem of lysine and lysatine and showed that the latter was actually arginine contaminated with lysine.

Following the elucidation of the mystery of lysine, lysatine and arginine, Kossel [83] demonstrated that the two amino acids, lysine and arginine, were

found in abundance in the basic protamines he had prepared from fish sperms. In 1897, Schulze and Winterstein [140] showed that ornithine and urea were among the decomposition products of arginine by alkalies. Jaffé [77] had previously discovered ornithine in 1878.

In 1910, Sörensen [145] synthesized arginine from benzoylornithine by condensation with cyanamide and subsequent hydrolysis of the benzoyl group with a strong acid. In 1924, Kossel and Gross [85] showed that arginine reacted with flavianic acid to form a very insoluble compound. Vickery [151] made use of this observation and developed an accurate, quantitative method for its estimation.

Iodogorgoic Acid (3,5-Diiodotyrosine)



In 1894, while at the Marine Zoological Station at Naples, Drechsel [31] investigated the amino acid content of the coral *Gorgonia Cavolinii*. The presence of iodine in this organism intrigued him. From the acid hydrolyzates of the skeleton of this organism, he isolated lysine, tyrosine and leucine. This convinced him that he was dealing with a protein. However, during the hydrolysis of the protein with hydrochloric acid, he observed the evolution of iodine. Thereupon, he altered his procedure and subjected the coral skeleton to barium hydroxide hydrolysis. After the removal of barium, he noted that samples of the hydrolyzates formed a heavy precipitate when treated with silver nitrate which, when boiled with strong nitric acid, led to the formation of the insoluble silver iodide. He added an excess of silver nitrate to the residue of his filtrate and the heavy precipitate which formed was recovered and treated with cold nitric acid. The insoluble silver iodide and sulfide were removed by filtration and the filtrate was neutralized with ammonia. Upon standing, a greyish precipitate formed and was recovered. It was dissolved in dilute hydrochloric acid and concentrated. Gradually there deposited crystals of a new compound — iodogorgoic acid. Drechsel's chemical analysis was inaccurate.

Prior to Drechsel's isolation of iodogorgoic acid, Hundeshagen [75] had analyzed many species of sponges for iodine and noted that they contained well over 10 per cent of this element. He communicated his findings to

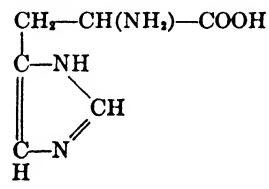
Drechsel, but unfortunately Hundeshagen was unable to crystallize iodogorgoic acid.

In 1903, Henze [69] repeated Drechsel's experiment, and with difficulty succeeded in isolating crystals of iodogorgoic acid from the protein of *Gorgonia Cavolinii*. He was the first to show that it gave a positive xanthoproteic test, thus indicating that he was dealing with an aromatic compound. He also reported accurate figures for the nitrogen and iodine contents of iodogorgoic acid. He observed that it did not react with Millon's reagent and that ortho-substituted tyrosine derivatives failed to give this test.

In 1905, Wheeler and Jamieson [156] reported on the synthesis of iodogorgoic acid. In 1907, Henze [69] confirmed their findings and showed that the iodogorgoic acid he and Drechsel had previously prepared was optically inactive, since their preparation was obtained from the barium hydroxide hydrolyzate of the protein.

In 1909, Oswald observed that the iodine of iodogorgoic acid is frequently removed during enzymatic digestion. Wheeler and Mendel [157] isolated this amino acid from the skeleton of the common sponge.

HISTIDINE



Histidine was discovered independently by two investigators. On April 9, 1896 Kossel [83] reported its isolation from the decomposition products of protamines; and on May 11, 1896 Hedin [68] isolated it from the acid hydrolyzates of proteins.

In 1894, Kossel began an investigation of protamines and observed that these products yielded heavy precipitates when added to solutions of soluble proteins. Kossel subjected sturin to sulfuric acid hydrolysis and removed sulfuric acid from the hydrolyzate with barium hydroxide. He next added mercuric chloride to the strongly alkaline solution. A heavy precipitate formed. This was recovered and treated with hydrogen sulfide. The mercury-free filtrate was concentrated. On standing, crystals of the chloride of a new base deposited. Kossel named this new compound histidine and accurately reported its chemical composition.

Hedin,[68] on the other hand, was investigating the fraction of acid hydrolyzate of casein that gave a copious precipitate with phosphotungstic acid and was studying its behavior toward silver nitrate. He decomposed the

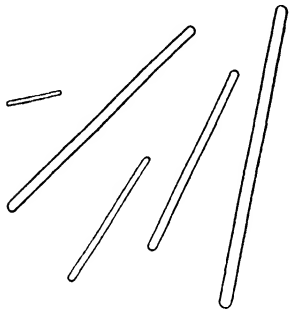
silver salts of the amino acid with a small amount of hydrochloric acid and removed the silver chloride formed. Following the concentration of the filtrate, he obtained a crystalline substance, the chemical composition of which was quite in agreement with that of Kossel's histidine. Hedin's method for the isolation of histidine is still in use.

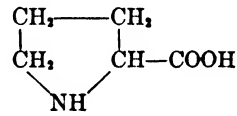
Histidine is widely distributed in nature. This was demonstrated by the extensive work of Kossel and his co-workers and by Schulze. Some of the peculiar properties of histidine observed at that time are noteworthy. Herzog [70] showed that it gives a biuret test and, upon boiling in a strong alkali, it yields hydrocyanic acid, ammonia, and carbon dioxide.

Pauly [116] demonstrated that histidine contains an imidazole ring which is responsible for its reaction with diazobenzenesulfonic acid and the development of a highly colored solution.

In 1911, Pyman [125] synthesized histidine and elucidated its structural formula.

PROLINE





The synthesis of proline was announced before its discovery among the decomposition products of protein hydrolyzates. It was synthesized in 1900 by Willstätter,[158] who became involved in an investigation on the chemistry of hygric acid (N-methylpyrrolidine-α-carboxylic acid). At that time there had been an argument as to whether or not the carboxyl group was in the alpha or beta position. Willstätter condensed sodium malonic ester with trimethylenebromide, then converted the bromopropylmalonic ester to the dibromopropylmalonic ester by treatment with bromine in the cold. He treated the resulting product with ammoniacal methyl alcohol to form the amide. By saponification with barium hydroxide he obtained α-pyrrolidinecarboxylic acid.

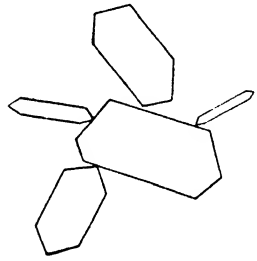
A year later, Fischer [49] synthesized proline from phthalimide propylmalonic ester. At that time Fischer did not know of Willstätter's discovery. Several other methods for the synthesis of proline have since been reported in the literature.

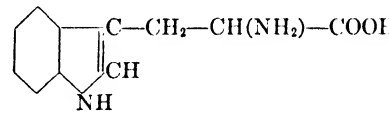
Credit for the discovery of proline among the decomposition products of protein digest belongs to Fischer, who in 1901 introduced his method

of fractional distillation of the esterified amino acids. He hydrolyzed casein with hydrochloric acid, esterified the amino acids and separated them by fractional distillation. One of the fractions, after saponification, was boiled with copper oxide. The copper salts of racemic proline separated. Fischer observed that the racemic copper salts of proline crystallized very readily, whereas the levo form yielded an amorphous mass in the mother liquor. After considerable research Fischer was able to prepare crystalline proline from an alcohol-ether mixture or from water by the careful addition of pyridine.

If one boils an aqueous solution of glutamic acid, there results a mixture of pyrrolidonecarboxylic acid and glutamic acid. Upon heating this mixture with an acid, glutamic acid is regenerated. It was, therefore, questionable as to whether proline was actually a constituent of proteins. Fischer and London [54] settled this argument by isolating proline from the enzymatic hydrolyzates of several proteins.

TRYPTOPHANE





As early as 1825, Tiedemann and Gmelin [148] reported the development of a violet color when chlorine was added to the pancreatic juice of a dog. This observation was made 76 years before the actual discovery of tryptophane.

Claude Bernard,[10] the discoverer of glycogen, became interested in Tiedemann and Gmelin's observations, probably on account of the similarity of their color test to that obtained on addition of iodine to glycogen. In 1856, he reported that the minced pancreatic tissue did not give this reddish color until putrefaction occurred. Minced liver, spleen and certain other glands behaved in a similar manner. Noteworthy was the observation he made at that time, that boiled pancreatic tissue lost its property of producing a reddish color with chlorine and that this color was apparently a property of proteins resembling casein. Bernard also studied the effect of bromine and iodine on pancreatic digests, but failed to obtain a color test. He was correct only with respect to iodine. In 1875, Kühne [87] introduced bromine water as a reagent for this substance. Kühne was also the first investigator to show that indole was not produced when digestion with trypsin was properly carried out, but only when putrefaction occurred. He was, therefore, the first to associate indole with

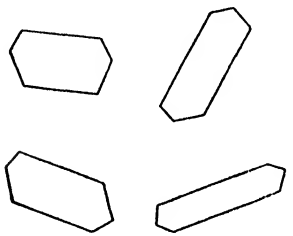
tryptophane. In 1890, numerous nvestigators attempted the isolation of the unknown chromogenic substance. Among them are: Neumeister,[107] Städelmann,[146] Nencki,[105] Beitler,[9] and others. However, it was Neumeister who proposed the name tryptophane, later adopted by Hopkins and Cole.

Another interesting color reaction of proteins was noted in 1874 by Adamkiewicz.[2] He observed that by the addition of concentrated sulfuric acid to a solution of albumin treated with glacial acetic acid, a violet color was obtained. Hopkins and Cole believed that the glacial acetic acid used by Adamkiewicz was probably contaminated with glyoxylic acid.

In 1901, Hopkins and Cole[73] discovered tryptophane in the enzymatic digest of casein. They first employed the glyoxylic acid test to trace the appearance of tryptophane and later as a guide in tracing it in their various fractions of casein digests. Their procedure consisted of adding 5 per cent sulfuric acid to the enzymatic digest of casein and one gram of mercuric sulfate dissolved in 5 per cent sulfuric acid to every gram of casein used. On standing for 24 hours, a yellowish voluminous precipitate formed which was filtered off and freed from tyrosine by washing it with dilute sulfuric acid. The complex formed was treated with hydrogen sulfide and barium hydroxide, and filtered. To the clear filtrate, sulfuric acid was added to precipitate the excess barium, and the solution was again filtered and acidified by the addition of 5 per cent sulfuric acid. Mercuric sulfate in sulfuric acid was next added until a slight precipitate formed. The turbid mixture was allowed to stand for half an hour and then filtered. This procedure removed most of the cystine. An excess of acidified mercuric sulfate was next added, which caused the precipitation of tryptophane as the mercury salt. After the removal of mercury, the filtrate was carefully concentrated in vacuum and mixed with alcohol. Tryptophane separated in a crystalline form. It was recrystallized from 75 to 80 per cent alcohol.

Ellinger[35] reported that tryptophane could have been the precursor of indole found in the intestine and previously noted in the putrefied digests of proteins. In 1907, Ellinger and Flammand[36] synthesized tryptophane from indolealdehyde by condensing the latter with hippuric acid, and showed that its structural formula was indole-α-amino-propionic acid.

ISOLEUCINE



$$CH_3 \diagdown CH{-}CH(NH_2){-}COOH \diagup C_2H_5$$

Leucine, isoleucine, and valine have great similarity to one another and

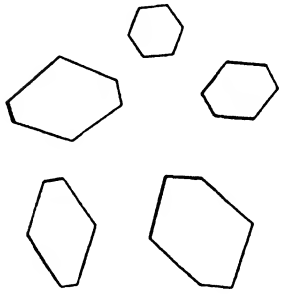
cannot be easily separated from aqueous or alcoholic solutions by simple fractional crystallization.

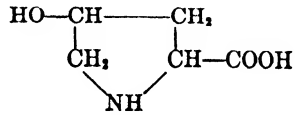
Isoleucine was discovered by Felix Ehrlich [33] in 1903. His early experimental work was carried out on beet molasses. He had known that the sirup contained a considerable amount of nitrogenous substances from which he was able to separate a material that showed the properties of a mixture of amino acids. From an alcoholic solution of this mixture he isolated what he at first thought was pure leucine. Chemical analysis of the crystals was also in agreement with that of leucine. However, it was more insoluble in water, possessed a higher specific rotation and a lower melting point. For comparison, he prepared its copper salt, and simultaneously the copper salt of pure leucine. The copper salt of pure *l*-leucine had been known to be very insoluble in water or in alcohol, but Ehrlich found that his material gave two copper salts, one being more soluble than the other. It occurred to him that he was dealing with an isomer of leucine.

While investigating the physical properties of the crystals, Ehrlich made the important observation that the copper salt of isomeric leucine was soluble in methyl alcohol. He was, therefore, able to isolate isoleucine, determine its specific rotation, and complete its chemical identification.

In 1906, Bouveault and Locquin [15] reported the synthesis of isoleucine from secondary butyl acetoacetic ester. Two years later, Ehrlich [34] published another synthesis in which he employed secondary butyl iodide as his starting material.

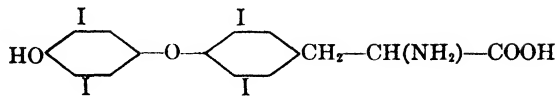
Hydroxyproline





In 1902, Fischer [50] isolated a new amino acid from the acid hydrolyzate of gelatin. On heating some of the crystalline compound with hydriodic acid and phosphorus, he obtained proline. He surmised its chemical structure. In 1905, Leuchs [90] synthesized this amino acid and named it hydroxyproline. Later Leuchs and his collaborators [91, 92, 93] established the position of the hydroxyl group and elucidated its structural formula.

THYROXINE



The discovery and synthesis of thyroxine has been one of the major developments of modern chemistry. In 1883, Kocher [82] showed that by the removal of the thyroid gland he was able to produce the symptoms of goiter. In 1891, Murray [104] prepared and used the first extract of thyroid to treat a patient suffering from hypothyroidism. In 1893, Ord and White [111] administered thyroid extracts and noted an increase in metabolism followed by greater urea excretion, a loss in body weight and a rise in body temperature. In 1895, Baumann [8] discovered iodine in the thyroid gland and suggested that it was present in a complex organic form analogous to that in which iron is present in hemoglobin. He published a method for the preparation of an extract rich in iodine and was of the opinion that potency was in direct ratio to the iodine present. He called his fraction iodothyrin. Baumann's discovery of iodine in the thyroid gland was indeed a great stimulus to chemists all over the world and led to the publication of numerous papers on the subject.

Roos [129] carried out an investigation on the fractionation of the active principle. He digested the gland with 10 per cent sulfuric acid, removed the insoluble matter and extracted the mother liquor with alcohol. The alcoholic extraction contained a considerable portion of the active principle. He termed this fraction thyroiodine. Oswald [115] worked out another procedure based on saline extraction of the gland and precipitation of the protein with saturated ammonium sulfate. The precipitate was dialyzed, redissolved in water and precipitated with alcohol. He called his fraction iodothyreoglobulin.

According to Hutchison,[76] neither thyroiodine nor iodothyreoglobulin contained a constant amount of iodine, or a constant amount of the active principle. Nürenberg [109] investigated the iodothyreoglobulin of Oswald and showed beyond any doubt that it was a protein. He conceived the idea that it might contain iodogorgoic acid, but was not successful in isolating it. He also investigated iodothyrin and found it to give a positive xanthoproteic reaction and negative Millon and Ehrlich tests. In this connection, one may recall that Henze [69] made a similar observation on iodogorgoic acid and pointed out that ortho-substituted tyrosine derivatives failed to

give Millon's test. Nürenberg concluded that the iodine is combined with tyrosine and possibly with tryptophane. Thereupon, he subjected iodothyreoglobulin to barium hydroxide hydrolysis for 30 hours and at the end of that time he removed the insoluble matter from the hydrolyzate and discarded it on the assumption that it was barium carbonate.

Since acid hydrolysis of the thyroid material did not produce satisfactory results, Kendall [80] found that severe alkaline hydrolysis was more advantageous and did not lead to the loss of the active principle. In his early experiments he hydrolyzed the glands for 24 hours in 5 per cent sodium hydroxide, separated the precipitate and noted its high iodine content and physiological activity. He subjected this fraction to additional hydrolysis for 18 hours in a mixture of sodium and barium hydroxides. An insoluble, iodine-containing precipitate was removed, and when the mother liquor was neutralized, a second precipitate separated. This fraction was very rich in iodine and was again hydrolyzed by the same procedure; once more another insoluble fraction containing 47 per cent iodine was obtained. The iodine-containing compounds were not completely dialyzable. Upon analysis, he found no traces of heavy metals. He dissolved the fraction containing 47 per cent iodine in 95 per cent alcohol and evaporated it on the water bath. Inadvertently, all the alcohol evaporated and the residue was brought to dryness. Instead of discarding it, Kendall added more alcohol to the hard crust and left it in the container. On the following day, he filtered it and recovered the solid matter. To his surprise this material contained 60 per cent iodine by weight and was very active. He suspended it in alcohol and dissolved it by adding sodium hydroxide. On neutralization of the solution with acetic acid, thyroxine crystallized in sheaves of needles.

Kendall's first yield of thyroxine amounted to about 18 mg. He repeated his experiment on a somewhat larger scale and secured approximately 200 mg of the crystals. Careful investigations of its physiological activity indicated that he was dealing with the active principle of the thyroid gland. He called his preparation thyroxine. In order to study its chemistry, Kendall needed a much larger quantity. It took him two years of research to obtain 33 grams from upward of three tons of glands and to learn that he had to use glass-lined equipment or nickel ware.

Misled by a nitrogen determination, Kendall concluded that thyroxine was a triiodo derivative of indolepropionic acid. Hick's [71] ultraviolet absorption spectra studies of thyroxine, tryptophane, and hydroxyindole propionic acid supported Kendall's hypothesis.

Harington [66] perfected a simpler method than Kendall's for the preparation of thyroxine. He hydrolyzed the thyroid glands with strong barium hydroxide. Whereas Kendall had reported a ratio of 3 : 1 of iodine to nitrogen, Harington found it to be 4 : 1, and reported the empirical formula of $C_{15}H_{11}O_4NI_4$. He subjected his crystalline product to dismutation and identified each fraction, thus determining the structural formula.

Harington and Barger [67] synthesized thyroxine and showed that it was identical with the natural product. Following the completion of their work, Harington and Barger submitted their paper for publication. Dakin had in the meantime completed his studies and arrived at substantially the same conclusion. He generously withdrew his communication. This was acknowledged by Harington and Barger.

Beta-Hydroxyglutamic Acid

Prior to 1942 this amino acid was considered one of the primary constituents of a large number of proteins. The recent work of Nicolet and Shinn [108] casts considerable doubt as to its existence in the hydrolyzates of proteins such as casein, lactalbumin, edestin, etc.

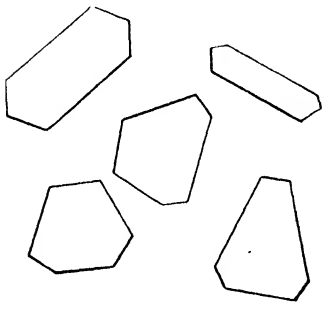
In 1918, Dakin [26] announced his well-known butyl alcohol method for the extraction of monoamino monocarboxylic acids and reported that the aqueous fraction contained the non-extractable dicarboxylic amino acids, among which there was a new substance which he separated and termed beta-hydroxyglutamic acid. He also reported its isolation, the preparation of its silver salts, its chemical structure and synthesis.

In 1934, Gulland and Morris [64] reported that they were unable to confirm Dakin's findings that casein contained 10.5 per cent beta-hydroxyglutamic acid. They estimated the amount to be about 0.3 per cent. In 1942, Nicolet and Shinn introduced their new periodic acid oxidation method for the estimation of the beta-hydroxy acids, serine and threonine, and pointed out that by their procedure they were unable to account for any beta-hydroxyglutamic acid in casein, lactalbumin and β-lactoglobulin. They stated: "It will be clear that if our results are accurate, there is no hydroxyglutamic acid in the hydrolyzate of the proteins here studied."

In 1941, Dakin reported that since he failed to convert the acid he isolated from casein into ketoglutaric acid or glutaconic acid derivatives, it seemed certain that his product could not be β-hydroxyglutamic acid.

According to Bailey, Chibnall, Rees, and Williams,[4] Dakin's acid was a mixture of degradation products of aspartic acid, cystine, and the hydroxy acids, serine and threonine. These investigators have shown that the treatment of hydrolyzates containing cystine with lime causes partial dismutation of the cystine molecule.

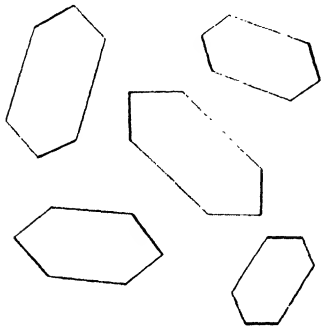
Methionine



$CH_3—S—CH_2—CH_2—CH(NH_2)—COOH$

Before the discovery of methionine, cystine occupied the focus of attention of biochemists investigating the sulfur-containing amino acids. In 1921, Mueller [100] reported that the hydrolyzates of certain proteins contained a substance essential for the growth of hemolytic streptococcus. His observations indicated that this factor was probably an unknown amino acid. Mueller continued his research on casein hydrolyzates and used his microorganisms as an indicator. He tested various chemical reagents and found that this unknown substance was precipitable by mercuric sulfate. Hopkins and Cole [73] had previously shown that during the precipitation of tryptophane by mercuric sulfate, some cystine and tyrosine were found in the precipitate. Mueller, therefore, tested cystine, tyrosine, tryptophane, and also histidine as growth stimulants for his cocci and found them to be ineffective. Following the fractionation of casein hydrolyzates with mercuric sulfate and the subfractionation of casein hydrolyzate with mercuric sulfate and the subsequent removal of mercury, he tried separation of the active principle from his filtrate by silver precipitation at a moderately alkaline reaction to litmus. The precipitate that separated was inactive. On concentrating the mother liquor, a crystalline substance deposited that possessed a remarkable growth-stimulating effect on streptococci. Upon chemical analysis, he found it to contain sulfur. The sulfur of this new compound did not behave like that of cystine for it did not react with basic lead acetate to give a black precipitate. In 1922, Mueller announced the discovery of a new sulfur-containing amino acid among the products of casein hydrolyzates. He showed that the nitrogen was present in the amino form and that its sulfur was very stable. In 1923, Mueller published an improved method for the preparation of the new sulfur-containing amino acid. Analysis of the crystalline product showed that it had a melting point of 280°–281° in a sealed tube and that its empirical formula was $C_5H_{11}SNO_2$. In 1925, Odake [110] investigated yeast extracts and was successful in isolating a substance that possessed the properties of Mueller's amino acid. In 1928, Barger and Coyne [5] synthesized it and after consultation with Mueller named it methionine. In 1930, Windus and Marvel [159] published another synthesis that proved to be more satisfactory.

Threonine



$CH_3—CH(OH)—CH(NH_2)—COOH$

The discovery of this amino acid was the outcome of the much-debated question of whether or not a mixture of pure amino acids could replace dietary proteins in meeting the nitrogen requirements of the growing animal.

In 1930, Rose and co-workers [130] investigated the basal requirements of the nitrogen components in the diet of rats. This investigation culminated in the isolation and chemical identification of the structure of a new amino acid found in casein hydrolyzates. In the course of their studies, it was discovered that the growth factor consisted of two components, one of which was identified as isoleucine. By increasing the isoleucine content in

Chemical Composition of Amino Acids

Amino Acids	Empirical Formula	M. W.	Chemical Composition C	H	O	N	
Alanine *l*(+)	$C_3H_7O_2N$	89.1	40.44	7.92	35.92	15.72	
Arginine *l*(+)	$C_6H_{14}O_2N_4$	174.2	41.36	8.10	18.37	32.16	
Aspartic acid *l*(−)	$C_4H_7O_4N$	133.1	36.09	5.30	48.08	10.52	
Cystine *l*(−)	$C_6H_{12}O_4N_2S_2$	240.3	29.99	5.03	26.63	11.66	26.7 (S)
Diiodotyrosine *l*(−)	$C_9H_9O_3NI_2$	433.0	24.96	2.09	11.08	3.23	58.6 (I)
Glutamic acid *l*(+)	$C_5H_9O_4N$	147.1	40.81	6.16	43.50	9.52	
Glycine (inactive)	$C_2H_5O_2N$	75.0	31.99	6.71	42.63	18.66	
Histidine *l*(−)	$C_6H_9O_2N_3$	155.1	46.44	5.84	20.62	27.08	
Hydroxyproline *l*(−)	$C_5H_9O_3N$	131.1	45.79	6.92	36.60	10.68	
Isoleucine *l*(+)	$C_6H_{13}O_2N$	131.2	54.93	9.99	24.39	10.68	
Leucine *l*(−)	$C_6H_{13}O_2N$	131.2	54.93	9.99	24.39	10.68	
Lysine *l*(+)	$C_6H_{14}O_2N_2$	146.2	49.30	9.65	21.89	19.16	
Methionine *l*(−)	$C_5H_{11}O_2NS$	149.2	40.24	7.43	21.45	9.39	21.5 (S)
Phenylalanine *l*(−)	$C_9H_{11}O_2N$	165.2	65.43	6.71	19.37	8.48	
Proline *l*(−)	$C_5H_9O_2N$	115.1	52.16	7.88	27.79	12.17	
Serine *l*(−)	$C_3H_7O_3N$	105.1	34.28	6.71	45.67	13.33	
Threonine *d*(−)	$C_4H_9O_3N$	119.1	40.33	6.61	40.29	11.76	
Thyroxine *l*(−)	$C_{15}H_{11}O_4NI_4$	776.9	23.19	1.42	8.24	1.80	65.3 (I)
Tryptophane *l*(−)	$C_{11}H_{12}O_2N_2$	204.2	64.69	5.92	15.67	13.72	
Tyrosine *l*(−)	$C_9H_{11}O_3N$	181.2	59.65	6.12	26.49	7.73	
Valine *l*(+)	$C_5H_{11}O_2N$	117.1	51.26	9.46	27.32	11.96	

their basal diet, Rose and his associates [160] were able to isolate the second component, determine its growth-stimulating action and its chemical composition. In 1935, Rose reported the discovery of this new indispensable amino acid. He termed it threonine on account of the relation of its chemical structure to the four-carbon sugar, threose. Its structural formula was established as alpha-amino-beta-hydroxybutyric acid.[163]

In 1925, Gortner and Hoffman [63] reported the isolation of a crude preparation containing 28 per cent ash and mentioned as a possibility that it might be hydroxyaminobutyric acid. In 1925–26, Schryver and Buston [134] described the finding of a new compound which they also believed to be hydroxyaminobutyric acid. They made no attempt to determine its chemical structure or its nutritional significance. In 1927, Rimington [126] published a paper on the preparation of hydroxyaminobutyric acid from phosphopeptone. He presented no evidence for its chemical structure.

The structural formula of threonine reveals the presence of two asym-

metric carbon atoms. Hence, it may exist in four optically active isomers. This was confirmed by Carter,[21, 164] who was the first to synthesize threonine. He separated the two racemic forms and resolved each into its optical isomer. One of these isomers proved to be identical with the natural product.

Threonine has since been reported to be present in a large number of proteins.

For a more detailed account of the discovery of the amino acids the reader is referred to the excellent treatise on the subject of "The History of the Discovery of the Amino Acids" by Vickery and Schmidt, *Chemical Reviews*, **9**, 169 (1931).

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Chapter II

Proteins: Occurrence, Amino Acid Content, and Properties

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Born in 1766 and died in 1828. He was the discoverer of palladium and rhodium and isolated cystine from a urinary calculus.

Introduction

The word *protein* is derived from the Greek word *proteios*, meaning first. It was used by Mulder [1] to designate the complex nitrogen-containing substances that are constituents of all animal and plant tissues. Mulder's notion that all proteins contain a common radical combined with sulfur and phosphorus, the amount of the latter elements varying with different proteins, has since been proved incorrect.

Proteins are constituents of all living tissues, the amount and kind of protein varying with the particular tissue in question. Proteins are so universally distributed that it is not possible to ingest naturally occurring foodstuffs without including protein, unless special means have been taken to exclude them. Together with water, inorganic salts, lipids, carbohy-

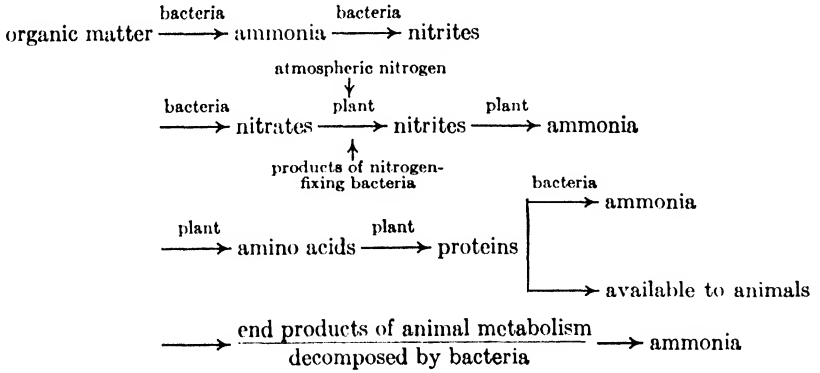
drates, vitamins, enzymes, etc., proteins constitute the materials that form the protoplasm of both animal and vegetable cells. Protoplasm should not be regarded as merely a mixture of the above components; rather it must be looked upon as a system in which these substances are in dynamic equilibrium, partly free and partly combined chemically, the whole functioning as a living machine capable of carrying on, under proper conditions, the normal metabolic activities of the cell and including such functions as maintenance, reproduction, and transfer of energy. Since proteins can combine with either hydrogen or hydroxyl ions, their state in living matter necessarily depends upon the pH of the system. The pH of animal tissues and fluids is generally maintained within comparatively narrow limits by means of buffer systems. The optimum pH, together with inorganic salts and water, permits solubility of some otherwise comparatively insoluble proteins. It also permits chemical combination with some of the other cell constituents to take place.

The proteins in the nuclei of both animal and vegetable cells are in large part combined with nucleic acid as nucleoproteins. The protein moiety — protamines and histones — is basic in nature and forms salt-like compounds with nucleic acid. The proteins of the cytoplasm belong to the class of albumins and globulins. Blood plasma contains albumin, globulin, and fibrinogen. The red blood corpuscles contain hemoglobin and a globulin. In certain of the invertebrates a copper-containing protein, hemocyanin, constitutes the oxygen-carrying vehicle. This is present in the circulating fluid rather than confined to cells. Various types of proteins are stored in special areas, such as in the seeds of plants. Here we find some proteins that are soluble in dilute salt solutions and others that are soluble in alcohol-water mixtures. Hair, wool, silk, horn, and hoofs contain very specialized proteins, the keratins, which are soluble only in solutions containing strong acid or alkali. The above-mentioned proteins differ from other proteins not only in their physical and chemical behavior but also in their content of amino acids. It should be emphasized again that proteins are almost universally distributed; therefore, unless means are taken to restrict its diet, the animal obtains its protein requirements from a large number of sources. Since proteins vary both qualitatively and quantitatively in their amino-acid content, and since it is essential that the animal be supplied with definite amounts of certain of the amino acids, variety in the sources and types of proteins ingested offers greater assurance that the necessary amino acids will be supplied than if the animal had to depend on a single protein with a rather limited or specialized amino-acid makeup.

Such enzymes as pepsin, pepsinogen, chymotrypsinogen, chymotrypsin, trypsinogen, trypsin, the various peptidases, urease, catalase, papain, amylase, lipase, maltase, etc. are protein in nature. In fact, it is highly probable that all enzymes are proteins. Those enzymic systems that participate in oxidation-reduction reactions, such as coenzyme 1, coenzyme 2,

and the yellow enzyme, which contain a vitamin in the prosthetic group, are essentially types of nucleoproteins. Many of the hormones — insulin, thyroglobulin, prolactin, parathyroid hormone, growth hormone, etc. — are proteins. The filtrable viruses are proteins. Those substances that are contained in or produced by bacteria and that engender the production of specific immune bodies are likewise proteins.

Plants constitute the ultimate source of proteins for the animal body. Even though the animal may ingest all or a large part of its protein requirements in the form of animal tissues and fluids, the ultimate source nevertheless is the plant. The plant can synthesize proteins from comparatively simple substances, such as nitrates, that are present in the soil solution; although there is evidence that nitrites and ammonia may also be utilized. According to some workers, there are also indications that higher plants can fix atmospheric nitrogen to some extent. Soil bacteria play a prominent rôle in rendering nitrogen available to the plant. The nitrite bacteria convert ammonia to nitrites; the nitrate bacteria, in turn, convert nitrites to nitrates. The symbiotic nitrogen-fixing bacteria are able to absorb atmospheric nitrogen and to convert it into nitrogenous compounds within their bodies, whence it ultimately becomes available to the particular plant on the roots of which these bacteria grow. Other non-symbiotic soil bacteria can also fix elementary nitrogen and thus render it available to the plant. Other soil bacteria have the ability to decompose nitrogen-containing organic matter with the production of ammonia; thus, together with the other reactions mentioned, a nitrogen cycle is established. We may represent this schematically: [2]



The mechanism of protein synthesis in the plant is far from having been elucidated. Presumably energy for the conversion of nitrates ⟶ nitrites ⟶ ammonia is provided by oxidation of carbohydrates. The easiest assumption is that ammonia combines with organic acids to form amino acids. In this assumption we are faced with the problem of the origin of the more complex ring-containing amino acids. Little is known about this subject. Asparagine and glutamine or both are synthesized in the higher

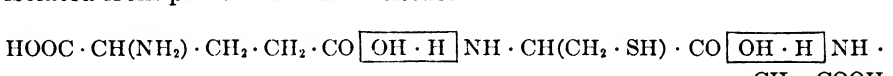
plants. This synthesis can also take place in the detached leaves, as has been shown in the case of rhubarb and tobacco leaves [3] and in etiolated seeds. The view has been expressed that the synthesis of these acid amides may be a mechanism for controlling the level of ammonia. It is possible that a certain amount of these compounds result from protein breakdown. On the other hand, there is evidence that *de novo* synthesis may occur, since the amounts of aspartic and glutamic acid present in the available proteins are insufficient to account for the amounts of the acid amides that are formed. The precursors of the acid amides are not known. As in the animal body, proteins are probably synthesized from amino acids in plants. Synthesis of amino acids and proteins may take place in the chloroplasts of the leaf cells due to the abundant supply of available carbohydrate resulting from photosynthesis, although such synthesis may take place in any of the growing cells of the plant if a supply of carbohydrate and of the proper nitrogenous compounds is available. Light is not directly essential to this process. The highest concentration of protein is present in the seeds. Other parts of the plant likewise contain protein. The alfalfa plant, for example, is quite rich in proteins. The seed proteins are probably formed from the amino acids present in the plant sap. The latter may result from hydrolysis of the proteins present in the leaves and elsewhere, or they may be transported directly to the seed from the places where they are synthesized without having gone through the protein stage. The available information regarding the synthesis of plant proteins is so meager that only a general and schematic idea of these processes can be presented at the present time. It is beyond the scope of this chapter to present and analyze all the views that have been offered on this subject.[2]

Neither is it the object of this chapter to discuss the details of the breakdown of proteins after entering the animal body and their ultimate transformation into the proteins that are present in and characteristic of the tissues and fluids of the particular animal in question. It suffices to state that through the agency of the various proteolytic enzymes present in the gastro-intestinal tract the ingested proteins are hydrolyzed to their constituent amino acids. These are absorbed into the blood stream and circulated to the tissues to be synthesized into the specialized proteins that characterize the various tissues. These proteins are specific not only for a given tissue but for the particular animal as well.

The proteins are exceedingly complex substances and it is not possible at the present time to write the structural formula of any protein as it is in the case of simple organic compounds. Mere mention of the fact that the molecular weights of proteins range from about 40,000 for egg albumin to 5,000,000 for hemocyanin (data based on ultracentrifuge measurements) indicates the enormous difficulty that confronts any one who might desire to represent the structures of proteins graphically. One fact stands out, and that is that proteins are composed of chains of amino acids linked

chemically. The amino acids may be considered the building stones of the protein molecule. To pursue the analogy further, we may consider the protein as a house that is constructed of different colored bricks, each of these bricks representing a particular amino acid. The architecture of the house is determined not only by the number of bricks used in its construction but also by their arrangement and by the number of each type that is used. It is easily seen that on the basis of the 22 amino acids that are now considered accepted (this term being used to designate those amino acids that have been isolated from proteins and their structures established), an almost infinite number of different protein molecules should exist. Fortunately, however, the number of known proteins is not as large as these possibilities would indicate.

We may represent the mode of linkage of amino acids in proteins by using as an illustration the simple peptide, glutathione, which has been isolated from plant and animal tissues:[4]



Glutamyl — cysteyl — glycine

Glutathione contains three amino acids: glutamic acid, cysteine, and glycine, chemically bound by the peptide (—CONH—) linkage. The chemical union has involved the loss of two molecules of water. The converse process, hydrolysis, involves the addition of two molecules of water, and thus the three constituent amino acids are set free. In proteins the amino acids are similarly joined. Proline and hydroxyproline contain an —NH but not an NH_2 group, and these amino acids are linked to other amino acids by the —CON— group instead of the more common —CONH— linkage. In proteins the number of each individual amino acid and the number of different amino acids is many times that indicated by the glutathione molecule. Moreover, in certain proteins, one or more of the 22 accepted amino acids may be lacking. A more detailed account of the structure of proteins is given in Chapter III. As will be shown in greater detail in Chapter IV, hydrolysis of proteins into their constituent amino acids may be carried out by (a) the action of appropriate proteolytic enzymes, (b) boiling with strong acids, (c) boiling with strong alkalies. Each of these methods possesses advantages as well as disadvantages.

Classification of Proteins

Since it is not possible to write the stereochemical structures, the classification of proteins must necessarily be somewhat arbitrary. The classification given below is largely based on physical properties, particularly solubility. This classification from many standpoints is very useful in that it gives the reader a general idea of the properties of the proteins that belong to each group, as well as a hint as to the general procedures that are

employed in isolating them and at times some information as to the mode in which they occur naturally.

Simple Proteins

Albumins. The albumins are soluble in water and are coagulated by heat at a slightly acid reaction (about pH 4.9). They may be thrown out of solution by saturation with ammonium sulfate. They are usually low or deficient in glycine.

Serum Albumin. Normal blood serum contains 4 to 6 per cent of serum albumin. In chronic glomerular nephritis this value may drop to 1.5 to 2.9 per cent; in nephrosis to 0.9 to 2.9 per cent. Serum albumin may be prepared from horse blood serum by first removing the serum globulins with half saturation of ammonium sulfate. The albumin fraction is precipitated at 62 per cent saturation with ammonium sulfate.[5, 6] At somewhat higher concentrations of this salt (68 per cent saturation), albumin, hemocuprein, choline esterase, glycoprotein, and phosphatase are precipitated. The ammonium sulfate can be removed by dialysis. Horse serum albumin has a molecular weight of about 72,000; that of man about 69,000 (in buffered salt solution). Its isoelectric point is 4.88. At pH 7.4, the reaction of normal blood serum, serum albumin is combined with cations (Ca^{++}, Na^{+}, etc.). Horse serum albumin has been fractionated into two portions, a crystalline albumin containing 5.5 per cent of carbohydrate and a carbohydrate-free albumin.[7] Human serum albumin is now being prepared in large quantities for intravenous administration in the treatment of shock. On the basis of molecular weights it is about three times more efficient in maintaining the colloidal osmotic pressure than is serum globulin (M.W. 175,000 in buffered salt solution).* The presence of serum albumin in urine may be demonstrated by addition of dilute acetic acid and heating. A cloudiness or flocculum appears, depending on the amount of albumin present. Strong acetic acid should not be added, since the protein will dissolve. Bence-Jones protein is soluble at high temperatures but precipitates at lower (room) temperatures.

Egg Albumin may be prepared from egg white by first removing the globulins with half saturation of ammonium sulfate. At pH 4.8 and on saturating the solution with this salt, the albumin is precipitated. The ammonium sulfate is removed from the precipitate by dialysis and the egg albumin is repeatedly recrystallized. Its molecular weight is 36,000 (in 6.66*M* urea solution) and its isoelectric point is 4.84 to 4.90.

Lactalbumin, the so-called albumin of milk, consists largely of lactoglobulin. It is quite insoluble in water but readily dissolves on addition of

* According to E. Mylon, M. C. Winternitz, and G. J. De Sütö-Nagy [*Am. J. Physiol.*, **139**, 313 (1943)], the therapeutic value of plasma in the treatment of shock is not fully explained by its colloidal osmotic pressure. Plasma albumin and globulin preparations are ineffective as compared with whole plasma in the treatment of shock in dogs.

salt solution.[8] β-Lactoglobulin has a molecular weight of 42,000. One mole of this protein contains 4 moles of cysteine, 4 of cystine, 9 of methionine, 2 of tryptophane, 9 of arginine, 21 of threonine, 15 of serine, 4–6 of histidine, 31–36 of lysine and 32 amide groups.[8a]

Globulins. The globulins are insoluble in distilled water but are soluble in dilute salt solutions and in dilute solutions of strong acids and alkalies. They are precipitable by addition of ammonium sulfate to half saturation.

Isolation procedures depend on precipitation of the globulin fraction of blood serum * with half saturation of ammonium sulfate and removal of the salt by dialysis. The globulins may be redissolved with the aid of small amounts of salt, reprecipitated and purified as indicated previously. They usually contain glycine.

Serum Globulin. The serum globulin content of normal human serum ranges from 1.4 to 3.0 per cent. It may be a little higher in nephritis and nephrosis. Both on the basis of fractionation with ammonium sulfate and electrophoresis it has been shown that serum globulin consists of at least three fractions, α-, β-, and γ-globulin.† Antitoxins are contained in the globulin fraction of serum. Guinea-pig complement consists of a euglobulin (C′1), a mucoglobulin which possesses both C′2 and C′4 activity, and a still uncharacterized component (C′3).[9] Horse serum globulin has a molecular weight of about 175,000 (in buffered salt solution). Its isoelectric point is 5.4 to 5.5. It is combined with cations in blood serum.

Tissue Globulin. The soft tissues of the animal body contain both albumin and globulin. Thus Luck [10] has reported that rat liver contains 5.1 to 7.9 per cent of globulin II, 3.0 to 4.5 per cent of euglobulin, 0.6 to 1.4 per cent of pseudoglobulin, and 0.7 to 1.9 per cent of albumin. The lower figures are for animals that were maintained on a low-protein diet and the higher values are for rats fed a high-protein ration.

One of the unique and interesting proteins is muscle globulin or *myosin*. It is prepared by extracting finely chopped fresh muscle with a solution of KCl and potassium phosphate of ionic strength ‡ 1.2 to 1.5 and pH 7 to 8.5. On increasing or decreasing the ionic strength of the filtered extract, precipitation of the protein occurs. The protein is insoluble at ionic strengths at which serum globulin is soluble. Myosin is insoluble at all

* Cohn and co-workers [*J. Am. Chem. Soc.*, **62**, 3396 (1940)] have described a method of fractionating blood plasma into 4 fractions by equilibration with alcohol-water mixtures of controlled pH, ionic strength, and temperature.

† γ-Globulin obtained from pooled human serum is being used to prevent or to ameliorate measles. Use is made of fibrin films as a substitute for brain covering and fibrin foam and thrombin are used to stop oozing of blood.

‡ The term *ionic strength* is defined as equal to the sum of the ionic concentrations of the electrolytes present in the solution, each ionic concentration being multiplied by the square of the ionic valence. The concentrations are expressed in gram ions per 1000 grams of solvent. The whole sum is divided by 2. See Lewis, G. N., and Randall, M., "Thermodynamics," p. 373, New York, McGraw-Hill Book Co., 1923.

salt concentrations between pH 5 and 6. When the acidity of the solution is greater than pH 5 or the alkalinity is greater than pH 10, the protein is soluble even in the absence of salt. Myosin solutions are very viscous. In the presence of small amounts of alkali and in the nearly complete absence of salt, muscle globulin forms gels. The gels are thixotropic.* Myosin solutions show double refraction of flow. This is due primarily to the orientation of the anisotropic † protein particles resulting from the shearing stresses that arise during flow. Photoelasticity also contributes to the phenomenon. Only undenatured myosin solutions show double refraction of flow, indicating that the phenomenon depends on the chemical nature and makeup of the protein molecule. Minimum combination with acids and bases takes place in the region pH 6.2 to 6.6.[11]

Anisotropic myosin fibers possess many of the properties of muscle fibers. The suggestion has been that the contraction of muscle is due to a sudden shortening of the long anistropic protein molecules in the fiber.[12] While this theory may not explain the phenomenon of muscle contraction *in toto*, it nevertheless suggests that the unique properties of muscle globulin may be a contributing factor. Muscle rigor represents a change of myosin to an insoluble form. Late evidence indicates that myosin is identical with adenosinetriphosphatase. This enzyme converts adenosinetriphosphate to adenosinediphosphate.[12a]

Vegetable Globulins. The proteins of seeds and nuts are in large part globulins. An extensive discussion of this subject is given by Osborne.[13] Edestin is a typical example of a vegetable globulin. It may be prepared by first extracting ground hemp seed with ether to remove lipids and chlorophyll, then triturating the meal with 10 volumes of 5 per cent sodium chloride solution at about 50°, and filtering. The extract is adjusted to pH 5.5 to 6.0 and dialyzed or permitted to stand at a low temperature. Crystals of edestin separate. They should be recrystallized several times, washed with distilled water, and dried. The molecular weight of edestin is about 49,500 (in 6.66*M* urea). Its isoelectric point is in the region of pH 5.5 to 6.0. Due to restrictions on the growth of hemp seed *Curcubita* seeds may be used.[14] The globulins from this source resemble edestin.

Prolamins. Proteins of this group have been isolated principally from cereal seeds. They are insoluble in water but are soluble in 70 to 80 per cent ethyl alcohol. On increasing the concentration of alcohol to about 90 per cent they become insoluble. The prolamins yield large amounts of

* Certain gels when shaken become fluid; on standing the gel again forms. This phenomenon is known as thixotropy.

† The reaction of gases, liquids, and some solids, when subjected to mechanical stress, is independent of the direction from which the stress is applied. Such substances are said to be *isotropic*. On the other hand, many substances including most crystals are *anisotropic*, *i.e.*, the resistance to stress depends upon the direction in which the stress is applied. In general this differs with the different axes of the crystal.

proline and amide nitrogen (due to the high content of glutamine) on hydrolysis. They are deficient in lysine. Typical proteins that belong to this group are zein obtained from maize, hordein from barley, and gliadin from wheat.

The isolation of proteins of this group may be illustrated by using gliadin as an example. Wheat flour is freed from starch by kneading it in running water. The moist gluten is repeatedly extracted with 70 per cent alcohol. The filtrate is concentrated in vacuo to a small volume. This is poured into 5 volumes of 1 per cent sodium chloride solution. The gliadin precipitates as a foam. This is dissolved in 70 per cent alcohol. On standing at low temperature most of the gliadin separates. This is subjected to further purification.[15] Gliadin forms a water-clear solution when dissolved in 50, 60, and 70 per cent alcohol. Its molecular weight is 40,000 (6.66*M* urea) and its isoelectric point is 6.5.

Glutelins. Proteins of this group are insoluble in water and in dilute salt solution but are soluble in the presence of dilute acids and alkalies. They represent heterogeneous mixtures of cell proteins after removal of the albumins, globulins, and prolamins. Glutenin from wheat and oryzenin from rice are members of this group.

Scleroproteins (albuminoids). These proteins are soluble only in solutions containing strong acid or alkali. The scleroproteins have a supporting or protective function in the animal organism, and in this respect they are analogous to cellulose and similar substances in the plant. It is doubtful that the isolated products are pure or homomolecular. Typical examples of proteins of this group are keratin from hair, hoof, and horn, fibroin from silk, and spongin from sponges.

Submembers of this group are: (1) *Collagens*, which are present in skin, tendons, and bones. They are converted into a water-soluble protein, gelatin, by boiling with water. The collagens are resistant to peptic and tryptic digestion. On the basis of the amount of glycine (G), proline (P), and hydroxyproline (HP) we may represent the structure of gelatin schematically by

$$—G \cdot P \cdot X \cdot G \cdot X \cdot X \cdot G \cdot HP \cdot X \cdot G \cdot X \cdot X—$$

where X represents other amino acids. Gelatin is deficient in valine, tryptophane, and tyrosine but rich in glycine, proline, hydroxyproline, arginine, and lysine. Its isoelectric point is 4.8 to 4.85. It is not homomolecular but contains a number of proteins with molecular weights ranging from 11,000 to 70,000. The purification procedure is essentially one that removes electrolytes by means of dilute acetic acid followed by water.[16,17] Gelatin is not antigenic.

(2) *Elastins* are present in such elastic tissues as the tendons and the arteries. They are not converted into gelatin, do not contain hydroxyproline, and are digested by trypsin.

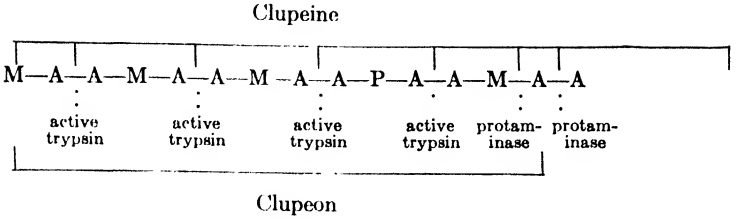
(3) *Keratins* are resistant to the action of pepsin and trypsin. The molecular ratio of histidine, lysine, and arginine is approximately 1 : 4 : 12. The cystine content varies from 0 to 16 per cent. Wool keratin may be prepared by dissolving wool in a solution of sodium thioglycollate at pH 12. The solution is filtered and, on addition of acetic acid, keratin precipitates. The semi-purified product is insoluble in water but soluble on addition of sodium carbonate or bicarbonate.[18] The solubilizing effect of potassium cyanide, alkaline sodium sulfide, and thioglycollic acid depends on the reduction of the disulfide groups of cystine which are essential for the maintenance of the fibrous structure.

Histones. Due to the basicity of this group of proteins they exist in the nucleated cells of animal tissues combined with nucleic acid (leucocytes, avian erythrocytes, and in the thymus, pancreas, kidney, etc.) or combined with a prosthetic group, such as heme in hemoglobin. The histones are water-soluble but insoluble in dilute ammonia. They are characterized by their high content of the basic amino acids, especially histidine and arginine.

Submembers of this group include *Globin*, the protein component of hemoglobin, which is obtained by adding hydrochloric acid and acetone-containing hydrochloric acid to an aqueous solution of carbon monoxide hemoglobin. A solution of heme and a precipitate of globin hydrochloride are obtained. On removal of the acetone the powdered globin dissolves in water. Free globin is obtained by quickly neutralizing this solution or by addition of ammonium sulfate to $\frac{1}{3}$ saturation.[19] Ox globin has a molecular weight of 37,000 (in buffered salt solution). The molecular ratio of tryptophane, tyrosine, arginine, histidine, and lysine in globin is approximately 2 : 3 : 3 : 8 : 9. Compound proteins such as globin cascinate [20] and globin insulinate [21] have been prepared.

Protamines are more basic than the histones and possess a more simple structure. They are essentially polypeptides. They occur in ripe fish sperm combined with nucleic acid. They are soluble in water, are not coagulated by heating, and are precipitated from aqueous solution by addition of alcohol. They form definite salts with strong acids. Protamines form compounds with acidic proteins such as insulin (protamine insulinate),[22] casein (protamine caseinate),[23] and edestin (protamine edestinate).[24] Typical protamines are salmine (from salmon sperm), sturine (from sturgeon sperm), clupeine (from herring sperm), scombrine (from mackerel sperm), and cyprinine (from carp sperm). On the basis of the content of basic amino acids, the protamines can be divided into those that contain (a) only arginine (monoprotamines), (b) arginine and lysine or arginine and histidine (diprotamines), and (c) arginine, lysine, and histidine (triprotamines).

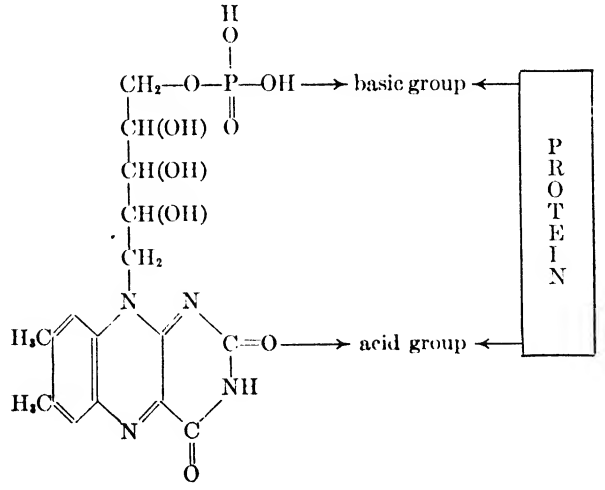
The action of proteolytic enzymes on clupeine may be represented schematically as follows:



Here M represents any one of four monoamino acids, P denotes proline, and A arginine. Of the 15 amino acids, 10 are arginine. Protaminase splits off two terminal arginine molecules yielding clupeon which, on being subjected to the action of trypsin, yields two dipeptides, A—M and M—A, and three tripeptides, A—M—A, A—M—A, and A—P—A.

Conjugated Proteins

This group includes those proteins that are combined with prosthetic groups of a non-protein nature. One member of this group, hemoglobin, has been well characterized; others are not yet well established. Protein complexes occur to a considerable extent in tissues. This is not unexpected when consideration is given to the amphoteric nature of the proteins and the types of free groups that are present in protein molecules. Functionally these compounds are very important. This is especially true of the coenzyme-protein compounds. These are a type of nucleoprotein containing vitamin or vitamin derivative, sugar, phosphoric acid, and protein. Several examples will serve to illustrate this. The yellow enzyme consists of riboflavin (ribose combined with alloxazine, a substituted pyrimidine), phosphoric acid, and protein as indicated in the following chemical formula:



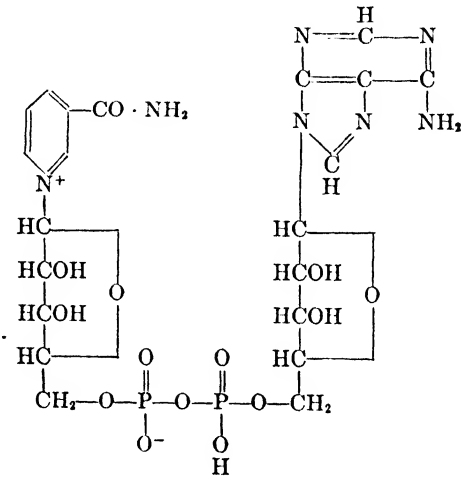
The yellow enzyme participates in oxidation-reduction reactions as illustrated by the following equations:

(1) Hexose-monophosphate + coenzyme 2 $\rightleftharpoons$ phosphohexonate + dihydrocoenzyme 2

(2) Dihydro-coenzyme 2 + yellow enzyme $\rightleftharpoons$ coenzyme 2 + reduced yellow enzyme

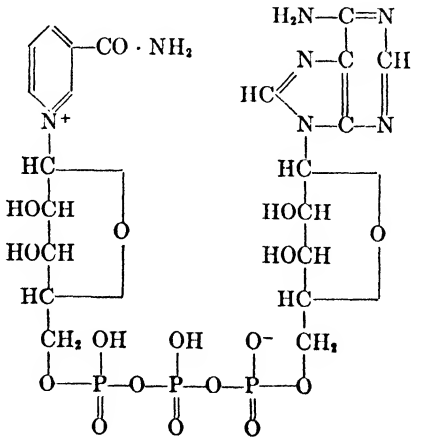
(3) Reduced yellow enzyme + $O_2 \rightleftharpoons$ yellow enzyme + H_2O_2

Coenzyme 1 consists of one molecule each of adenine and nicotinic acid amide and two molecules each of phosphoric acid and *d*-ribose as shown below:



On reduction this forms dihydro-coenzyme 1 by acceptance of two hydrogen atoms.

Coenzyme 2 consists of one molecule each of nicotinic acid amide and adenine, two molecules of *d*-ribose, and three molecules of phosphoric acid, as indicated by the following chemical formula:



Coenzyme 2 can accept two atoms of hydrogen and hence also participates in oxidation-reduction reactions. Both coenzyme 1 and 2 are combined in nature with proteins.

Carboxylase contains magnesium, thiamine diphosphate, and protein. Its action is to split carbon dioxide from pyruvic acid to yield acetaldehyde:

$$\begin{array}{l} CH_3 \\ | \\ C{=}O \\ | \\ COOH \end{array} \longrightarrow CH_3 \cdot CHO + CO_2$$

A more detailed discussion of enzymes of this type is given by Sumner and Somers.[25]

Very little is known about the nature of the proteins that occur combined with coenzymes or the mode of union between the protein and the prosthetic groups. The following are possibilities: (a) acid-base type of combination; (b) combination between the OH-group of phosphoric acid and the NH_2-group in the guanidino moiety of arginine yielding the $\begin{array}{c} | \\ -N- \\ | \end{array}\!\!\begin{array}{c} | \\ P{=}O \\ | \end{array}$ linkage, although combination of phosphoric acid with other amino groups is not excluded.

It is to be especially emphasized that in biological systems, because of their amphoteric nature and of the number and types of free reactive groups, proteins in general must not be regarded as existing in the free state, but rather in the form of complexes, this term being used to imply both firm and loose combination between proteins and other cell constituents and including both anions and cations. Further characterization of conjugated proteins is given below.

(a) **Nucleoproteins.** As indicated previously, nucleoproteins consist of nucleic acid or nucleic acid-like compounds combined with proteins. They may be prepared by extracting nucleated tissues with a dilute solution of alkali. Dissociation of nucleic acid from the protein may be effected by treatment with cold mineral acids. One of the most interesting groups of nucleoproteins is contained in the chromosomes. They appear to be arranged according to a definite pattern. The basic groups of the protein lie on or near the surface and are combined with nucleic acid. The genes probably contain considerable amounts of nucleoproteins. It is quite definitely established that the filtrable viruses are nucleoproteins.[26] The nucleic acids of the plant virus are of the yeast nucleic acid type, *i.e.*, the carbohydrate is *d*-ribose. Curiously eastern equine encephalomyelitis and influenza A virus also contain the plant type of nucleic acid. Tobacco mosaic virus has a molecular weight of 43,000,000 and rabbit papilloma and eastern equine encephalomyelitis each have a molecular weight of about 20,000,000. Tobacco mosaic, tobacco ring spot, and rabbit papilloma viruses are composed of amino acids and nucleic acid only. Arginine,

aspartic acid, cysteine, glutamic acid, leucine, lysine, phenylalamine, proline, serine, tryptophane, and tyrosine have been isolated from the protein of tobacco mosaic virus. The presence of alanine, histidine, and glycine have not yet been demonstrated. Latent mosaic virus may contain a conjugated polysaccharide. Eastern equine encephalomyelitis virus appears to contain phospholipids, cholesterol, and fatty acids in addition to nucleoprotein; vaccine virus contains also phospholipids, cholesterol, and fat. The viruses are an exceptionally interesting group of substances in that when placed on the proper substrate they reproduce their kind. These molecules can be subjected to various chemical treatments without losing their inherent properties.

(b) **Glycoproteins.** Many of the naturally occurring proteins contain carbohydrate or derivatives of carbohydrates as part of the molecule. Among these are glucose, lactose, mannose, glucosamine, galacturonic acid, aldobionic acid, etc. Immunological specificity of the glycoproteins depends on the specific carbohydrate combined with the protein. Meyer [27] has proposed the following classification of the glycoproteins:

A. MUCOPOLYSACCHARIDES
   I. *Containing uronic acid*
      (a) Sulfate-free
         1. Vitreous humor, umbilical cord, synovial fluid, group A streptococcus
      (b) Sulfate-containing
         1. Chondroitinsulfuric acid
         2. Mucoitinsulfuric acid (gastric mucin and cornea)
         3. Heparin
   II. Neutral mucopolysaccharides of known composition
      (a) Chitin
      (b) Gastric polysaccharide
      (c) Bacterial polysaccharide

B. GLYCOPROTEINS containing neutral mucopolysaccharides of unknown composition:
      (a) Ovomucoid-α (formerly termed ovomucoid)
      (b) Ovomucoid-β (formerly termed ovomucin)
      (c) Serum mucoid, serum glucoid
      (d) Globulins (egg white, thyroglobulin)
      (e) Pregnancy urine hormone

(c) **Phosphoproteins.** These proteins differ from nucleoproteins in that they contain phosphoric acid but no carbohydrate, purine, or pyrimidine group. The phosphoric acid is present in ester combination with hydroxy amino acids and especially with the hydroxy group of serine. Two repre-

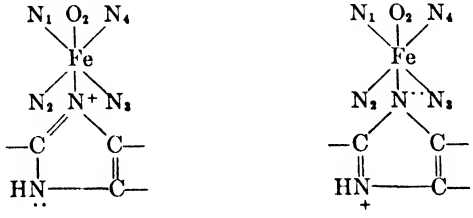
sentatives of this group are casein (present in milk) and vitellin (present in egg yolk). Neither appears to be homomolecular.

Nutritionally and industrially, casein is one of the most important proteins. It occurs in milk combined with calcium as calcium caseinate. The action of rennin, an enzyme present in the stomachs of young animals, is to convert casein into paracasein, the calcium salt of which is insoluble. Casein is usually isolated from fat-free milk by adding a dilute solution of a mineral acid to pH 4.6, the isoelectric point of casein, washing the precipitated casein with distilled water, redissolving it with the aid of dilute sodium hydroxide, reprecipitating as before, washing the casein free from salts, removing the water by washing with alcohol, removing the latter with ether, and drying the product. The molecular weight of casein is 33,600 (in 6.66*M* urea).

Because of its low content of the sulfur-containing amino acids, casein is unable to meet the amino-acid requirements of growing rats unless it is fed at a level of 18 per cent or above, or it is supplemented with methionine. Industrially casein finds extensive application in the manufacture of glues, paints, and plastics, and in the sizing of paper.[28]

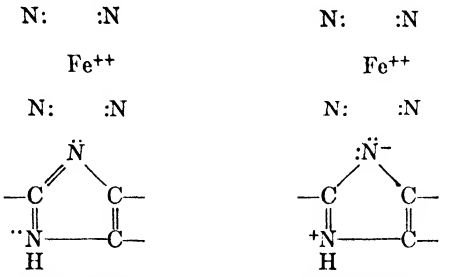
(d) **Chromoproteins** are characterized by the presence of a heavy metal (iron, copper, manganese, vanadium, cobalt, magnesium, etc.) in the molecule. They may also contain a prosthetic group, particularly a porphyrin group.

Hemoglobin is the best characterized member of this group of proteins. It constitutes the oxygen-carrying vehicle of mammalian blood. Approximately one gram of hemoglobin will combine with 1.36 cc of oxygen. The iron content is about 0.33 to 0.34 per cent which corresponds to a minimum molecular weight of about 16,000. Estimation of the molecular weight of hemoglobin in aqueous solution gave a value of about 67,000, or about four times the calculated minimum molecular weight. The structure of the iron-containing prosthetic group, heme, is evident from its derivative, hemin, obtained by dissociating heme from hemoglobin by the action of HCl. There are four heme groups in hemoglobin; each contributes two carboxyl groups to the acid groups of hemoglobin. The bonds around the iron may be represented schematically by



Resonating Forms of Oxyhemoglobin where N_1, N_2, N_3, and N_4 represent the pyrrole nitrogen atoms of the porphyrin nucleus and the ring structure histidine

As indicated in the above formulas, the iron is bound to the globin through the imidazole group of histidine. The two forms of reduced hemoglobin may be represented schematically by



Resonating Forms of Reduced Hemoglobin

The chemical formula of carbon monoxide hemoglobin is similar to that of oxyhemoglobin. In both cases the bonds that unite the iron atom to the six surrounding groups are covalent. Carbon monoxide is much less easily dissociated from hemoglobin than is oxygen.

Methemoglobin is formed when hemoglobin is treated with an oxidizing agent such as potassium permanganate, the chlorates, nitrobenzene, as well as with pyrogallol, acetanilide, and other compounds. The iron in methemoglobin is in the ferric state, Fe^{+++}, whereas in oxyhemoglobin it is in the ferrous condition, Fe^{++}. Methemoglobin appears in both blood and urine when compounds such as the above are taken into the body.

Due to the ease with which it may be crystallized, hemoglobin is best prepared from horse blood. The red corpuscles are repeatedly washed with isotonic salt solution to remove serum proteins, the corpuscles are laked by addition of distilled water, the stromata are removed by centrifuging, and the hemoglobin is crystallized at 0° after oxygenation or treatment with carbon dioxide. Addition of alcohol up to a concentration of about 20 per cent facilitates crystallization.[29] The isoelectric point of horse hemoglobin is 6.78.

Muscle hemoglobin.[30] Of the total hemoglobin present in the body, about 25 per cent is contained in muscle fibers and does not circulate. This fraction is termed muscle hemoglobin, or myohemoglobin. The highest concentrations occur in those muscles whose complete cycle of activity is one or more seconds and whose action must be maintained over long periods of time. Examples of such muscles are the heart muscles of larger animals, the breast muscles of flying birds, and the leg muscles of running animals. Muscles that contract several times a second (wing muscles of flying insects) and muscles that contract intensely with a large interval of time between contractions contain little or no muscle hemoglobin but considerable percentages of cytochrome.

The oxygen dissociation curve of muscle hemoglobin is hyperbolic; that of blood hemoglobin sigmoid in shape. Muscle hemoglobin has a much

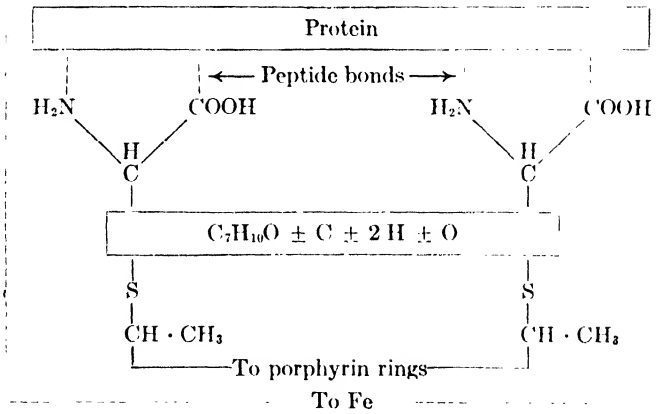
higher affinity for oxygen than blood hemoglobin. Thus at 40 mm oxygen pressure (venous pressure) the amount of oxyhemoglobin in muscle hemoglobin is about 95 per cent, whereas in blood hemoglobin it is 66 per cent. In the case of muscle hemoglobin only about 5 mm of oxygen pressure is necessary to yield 66 per cent of oxyhemoglobin. Since the affinity of muscle hemoglobin lies between that of blood hemoglobin and the oxidases, it is well adapted to serve as an intermediary for taking up oxygen from one compound (blood hemoglobin) and giving it up to the other compound (oxidases). Due to extremely great affinity for oxygen, the oxidases can function at oxygen pressures below 5 mm. At this pressure muscle hemoglobin has given up about 40 per cent of its oxygen and the oxyhemoglobin content of blood hemoglobin is about 5 per cent. Under physiological conditions muscle hemoglobin can take up and give off oxygen very rapidly, the times for the half reaction being 0.001 and 0.01 second respectively. Muscle hemoglobin contains only one iron atom per molecule. Its molecular weight is 17,500 and the isoelectric point (horse muscle hemoglobin) is 6.99. Details for the preparation of this protein are given by Theorell.[31]

Ferritin[32] is an iron-protein compound that occurs in the liver, spleen, and marrow of mammals. It serves as a storage compound of iron. Ferritin consists of a protein fraction, apoferritin, which is linked together with a special type of colloidal ferric hydroxide, the amount of iron being about 23 per cent. The iron in the micelles of ferritin is present in the state of three unpaired electrons per atom of iron which makes it possible to distinguish it magnetically from any other form of ferric iron. When, for example, ferric ammonium citrate (5 unpaired electrons per atom of iron) is injected into a dog, the iron is converted into ferritin iron (3 unpaired electrons per iron atom). The iron resulting from the breakdown of hemoglobin is converted, at least in part, to ferritin iron.

A few other proteins that contain iron in the prosthetic group are: peroxidase (Fe-protoporphyrin), catalase (Fe-protoporphyrin plus bile pigment hemochromogen), and cytochrome c. The latter compound is of special interest and warrants some consideration here. It is widely distributed in tissues. *Cytochrome c* is a heme-protein, but it differs from hemoglobin in that it contains only one heme per molecule. Its molecular weight is about 13,000. It contains about 25 per cent lysine and 3.3 per cent histidine. The number of free amino and carboxyl groups suggests that the molecule consists of several polypeptide chains with free amino and carboxyl groups at each end. Two of the three imidazole groups in cytochrome probably constitute the hemochromogen-forming groups. The heme of the cytochrome is built into the protein moiety by means of thioether linkages from the side chains of the porphyrin to the protein, and by means of the two imidazole groups of histidine which are strongly bound to the iron atom on each side of the flat heme disc. The heme group is

probably built into a "crevice" of the protein molecule.[33] Since oxygen cannot approach the iron atom, cytochrome c is not autoöxidizable. Within physiological limits of pH it cannot form compounds with carbon monoxide or cyanides. In living cells oxidation of cytochrome is brought about by cytochrome oxidase, another heme-protein. This is probably identical with Warburg's respiration enzyme. The iron is oxidized from the ferro to the ferri state. The reduction of cytochrome is catalyzed by cytochrome reductase. Cytochrome differs from hemoglobin in its low content of histidine. Moreover, in hemoglobin, one imidazole group is in a favorable position for coordination with the iron atom, but the other is not. Thus one of the six octahedral valences of the iron atom is left free for the addition of oxygen, carbon monoxide, etc. As indicated previously, such combination is not possible in cytochrome.

The structure of cytochrome may be represented schematically as follows:[34]

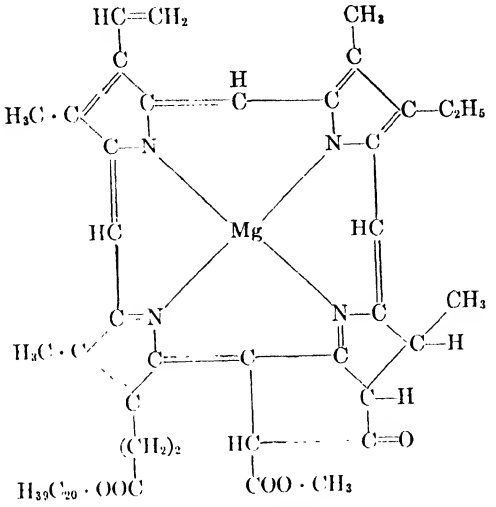


The $C_7H_{10}O$ compound appears to be a tertiary cyclic nitrogen-containing base that is combined with porphyrin in the 2- or 4-position with the formation of a quaternary ammonium salt. The sulfur bridges appear to be cystine.

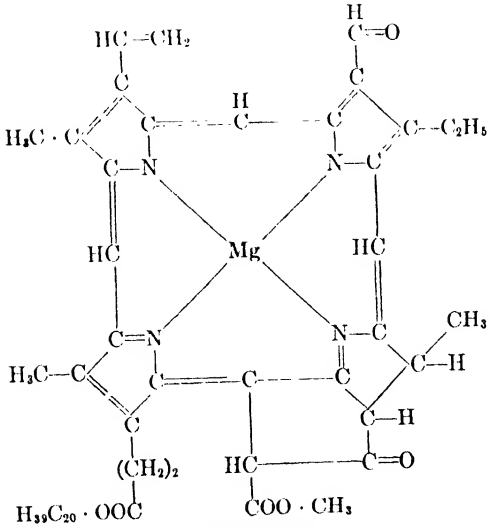
The oxygen-carrying agent of certain of the lower invertebrates — crab, snail, octopus, and squid — is a copper-containing protein, *hemocyanin*. Unlike hemoglobin it is present in the circulating fluid and not in cells. In the oxygenated condition hemocyanin is blue; in the reduced form it is colorless. The molecular weights — 400,000 to 6,000,000 — indicate that there are a number of hemocyanins. Some of the larger molecular weights may be indicative of extensive aggregation of smaller units. In *ascidians* a vanadium-containing protein functions as the oxygen-carrying vehicle.

Other examples of copper-protein complexes are laccase, tyrosinase, ascorbic acid oxidase, polyphenol oxidase, turacin, hemocuprein, and hepatocuprein.

The classical example of a magnesium-containing prosthetic group is furnished by the chlorophylls, the green coloring matter of plants. There are several chlorophylls, the best characterized ones being α- and β-chlorophyll whose structures are given below:



α-Chlorophyll



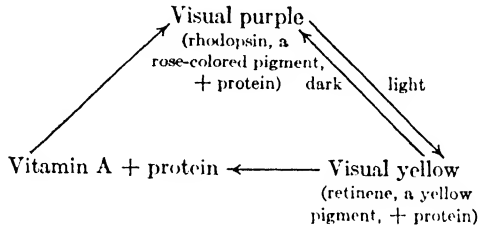
β-Chlorophyll

In the above formulas phytol, $C_{20}H_{39}OH$, an unsaturated alcohol, is combined as an ester. Chlorophyll is combined with protein in plant tissues.

It has been shown previously that carboxylase is a diphosphothiamine magnesium-protein complex. The purest preparation contained 0.46 per cent of diphosphothiamine and 0.13 per cent magnesium. It is not certain

how the prosthetic group is combined with the protein. It is possible that magnesium forms the connecting link although the possibilities that phosphoric acid may be the connecting bridge or that combination may take place through the quaternary nitrogen of the coenzyme are not excluded.

Other less well characterized chromoproteins are leucokeratin and melanokeratin, which are present in hair. Blond hair and finger nails contain leucokeratin, and brown hair contains largely melanokeratin and some leucokeratin. Red hair contains rhodokeratin. This substance contains iron. Ovoverdin, which occurs in the eggs of the lobster, is a carotenoid protein. Its molecular weight is 300,000 and its isoelectric point is 6.7. The carotenoid proteins play a special rôle in vision. Visual purple occurs in the rods of the eyes of vertebrates. It is a carotenoid-protein compound. We may represent the function of visual purple in vision as follows:



The action of light on visual purple gives rise to visual yellow; in the dark the reverse reaction occurs. In vitamin A deficiency an insufficient amount of visual purple is formed and night blindness occurs. It is not known how vitamin A is combined with the protein. In marine fishes rhodopsin and in fresh-water fishes porphyropsin have functions similar to that of visual purple.

Metalloproteins. All heavy metals are capable of forming complex ions with proteins. A simple method of determining the presence of metalloprotein ions is by electrical transport. If, for example, a solution of calcium caseinate is placed in a three-compartment cell with platinum electrodes and a direct current is passed through the solution, it is found that some of the calcium ions have migrated to the negative pole and some of the casein ions to the positive pole. However, a certain number of negatively charged calcium caseinate ions have migrated to the positive pole. The latter are of the nature of complex metal-protein ions. Such ions occur extensively in biological fluids. For example, on filtering blood serum through a collodion bag, about 50 per cent of the calcium is filtrable; the remainder is not. The filtrable fraction represents inorganic calcium compounds, chiefly calcium phosphate, and is quite highly ionized. Its level is maintained by the parathyroid hormone. In hypoactivity of the parathyroid gland the level of filtrable calcium drops and tetany usually results. On administration of parathyroid hormone by injection, the filtrable calcium fraction is increased. When the level becomes so high that the solu-

bility of calcium phosphate is exceeded, colloidal calcium phosphate is formed, and this is non-filtrable. The non-filtrable calcium fraction represents calcium-protein complexes. The calcium is combined chiefly with serum albumin. In nephritis, due to loss of serum albumin, this fraction of calcium is decreased. When metallic electrodes can be employed, the presence of metal-protein complex ions may be demonstrated by measurement of the activity of the metallic ion. Silver electrodes, for example, may be so employed. In the presence of protein the activity of the metallic ion is decidedly less than in a solution of the metallic salt from which protein is absent.

Some of the heavy metal-protein complexes are of practical importance. Thus compounds of silver with casein, gelatin, egg albumin, and serum albumin find extensive use as mild anti-infective agents.[35] These solutions are decidedly less caustic than, for example, a solution of silver nitrate. Zinc * protamine insulinate is commonly used in the treatment of diabetes. Insulin containing radio-active zinc has been prepared.[36]

Lipoproteins are not as yet well characterized. Lecithinoproteins probably exist.[37] On the basis of the zwitterion structure of the phospholipids, such compounds may be formed, although this will depend on the particular phospholipid and the pH of the solution. Lecithin, cephalin, and sphingomyelin may conceivably be combined with proteins through the $\rangle N—P=$ linkage. Definite proof for this is lacking.

Derived Proteins

By the action of hydrolytic agents such as acid, alkali, and proteolytic enzymes, under appropriate conditions, proteins may be partially hydrolyzed. The products thus formed, except for the simple peptides, are not well characterized from a chemical standpoint. Simple peptides are usually prepared synthetically, although a number have been isolated from naturally occurring sources or from partial protein hydrolyzates. The formation of protein derivatives may be schematically represented as follows:

Protein ⟶ primary proteose ⟶ secondary proteose ⟶ peptone ⟶ polypeptides ⟶ simple peptides ⟶ amino acids.

It should not be inferred from the above that in the earlier stages of protein hydrolysis one or more amino acids may not be set free. There is ample evidence that during digestion of proteins certain amino acids are selectively liberated.

When only slight changes are induced in the native protein, the early derivative resembles the parent substance with respect to such properties

* Most of the zinc is probably present as zinc phosphate.

as coagulability and salting out. Later these properties are lost. The primary proteoses are precipitable by half saturation with ammonium sulfate, the secondary proteoses only by complete saturation with this reagent. The peptones remain soluble under these conditions. They are precipitable by phosphotungstic acid. As hydrolysis proceeds, antigenic proteins lose their antigenic properties. The higher proteoses are usually toxic; the smaller molecules are non-toxic. Proteoses and peptones result from the action of pepsin on native proteins, although small amounts of amino acids may also be formed. Pepsin does not hydrolyze the keratins, mucines, protamines, and most peptides, although exceptions have been found. Some simple peptides that have been isolated from the partial decomposition products of proteins together with their sources are: glycyl-*d*-alanine (silk fibroin), glycyl-*l*-leucine (elastin), glycyl-*l*-tyrosine (silk fibroin), *d*-alanyl-glycine (silk), *d*-alanyl-*l*-leucine (elastin), and *l*-prolyl-*l*-phenylalanine (gliadin). Valylvaline has been isolated from *gramicidin* hydrolyzates.[39]

Proteins of Endocrine Origin. It has become increasingly evident that many of the endocrine products of animals are protein in nature. This makes it necessary that they be administered parenterally in order to obtain the desired physiological response, since on oral administration they are digested and thus their specific properties are lost. A possible exception to this statement is *thyroglobulin*, the hormone of the thyroid gland. Its action depends on the presence of the amino acid thyroxine in the molecule and hence on oral administration of thyroglobulin its activity is not destroyed. However, there are opinions that there are some differences in the effects produced by thyroxine and by thyroid hormone. Some of the amino acid percentages reported on thyroglobulin are: cystine, 4.3; methionine, 1.3; tryptophane, 1.9; tyrosine, 3.0; diiodotyrosine, 0.7; thyroxine, 0.3. The content of glucosamine is 2.2 per cent. Administration of thyroglobulin to normal animals leads to an increase in basal metabolism; a deficiency in this hormone is characterized by a sub-normal basal metabolism.

Insulin is the carbohydrate-regulating hormone secreted by the islet cells (islets of Langerhans) of the pancreas. Removal of the pancreas or deficiency in the amount of hormone secreted is followed by hyperglycemia and glycosuria. The condition is termed diabetes. Treatment involves replacement therapy. Administration of insulin to normal animals leads to hypoglycemia. When the blood-sugar level is sufficiently low convulsions result. Injection of epinephrine into such animals is followed by an elevation of the blood-sugar level by the conversion of liver glycogen to glucose. The following percentages of amino acids in insulin have been reported: leucine, 30; tyrosine, 12.5; arginine, 3; histidine, 4; lysine, 2; glutamic acid, 20; cystine, 12.5; serine, 3.6; threonine, 2.7; proline, 10; and phenylalanine, 1. Zinc is present to the extent of 0.5 per cent. The molecu-

lar weight of insulin is about 35,100 and its isoelectric point is 5.35. The preparation of crystalline insulin is given by Sahyun.[39]

The hormone of the parathyroid gland (*parathormone*) appears to be protein in nature. A single pure product has not yet been isolated. The most recent attempt to isolate the hormone [40] yielded two components, one having a molecular weight of about 20,000 and the other a molecular weight of 500,000 to 1,000,000. The active principle is inactivated by acid and basic hydrolysis and by digestion with proteolytic enzymes, especially by pepsin. Ultraviolet absorption studies show the presence of tyrosine, phenylalanine, and tryptophane. Acetylation with ketene leads to inactivation of the hormone. There are indications that the lower molecular weight fraction is responsible for the activity of this hormone. As indicated earlier, the parathyroid hormone functions to regulate the level of the filtrable calcium fraction of blood serum.

The anterior lobe of the pituitary gland secretes at least six hormones that have received attention. These are the adrenocorticotropic, thyreotropic, lactogenic, growth, follicle-stimulating, and interstitial cell-stimulating. All of these appear to be proteins. Several have been prepared in reasonably pure form. The *adrenocorticotropic hormone* [41] has a molecular weight of 20,000 and its isoelectric point lies between 4.7 and 4.8. It is stable at 100° in a pH 7.5 buffer solution and in 0.1*M* HCl but not in 0.1*M* NaOH. It is quite stable to peptic digestion (37 per cent) but its activity is destroyed by tryptic digestion and by trichloroacetic acid. On the basis of electrophoretic and ultracentrifugal studies, this protein behaves as a homomolecular substance. The method of isolation is described in the references cited. This hormone stimulates the adrenal cortex to produce or discharge its hormone.

Prolactin has been prepared in crystalline form.[42] On the basis of electrophoresis, ultracentrifugation, and solubility measurements, the product is homomolecular. Its isoelectric point lies between 5.65 and 5.70. The molecular weight is in the neighborhood of 35,000. The following percentages of amino acids have been reported: tyrosine, 5.5; tryptophane, 1.3; cystine, 3.4. The cystine content accounts for about 45 per cent of the total sulfur content of this protein. The biological activity of prolactin is destroyed by both peptic and tryptic digestion before the hydrolysis has proceeded to the point that the hydrolytic fragments are no longer precipitable by trichloroacetic acid. In this respect prolactin resembles insulin. The hypoglycemic activity of the latter hormone is rapidly destroyed by pepsin. Prolactin is concerned with the onset of lactation in mammals, growth of the crop gland of pigeons, initiation of broodiness in the fowl, and marked diminution in the size of the active gonads of adult pigeons or fowl.

Although a highly potent preparation of the *growth hormone* has been reported,[43] the product cannot as yet be considered as meeting the require-

ments of a pure protein.* The hormone appears to be a globulin. A non-crystalline protein having marked *thyreotropic* activity has been isolated from beef pituitary glands.[44] On the basis of electrophoresis, the product is homogeneous and of relatively low molecular weight. The thyreotropic hormone stimulates the thyroid gland with consequent elevation of the basal metabolic rate. The *interstitial cell-stimulating (luteinizing) hormone* of swine pituitaries is a protein having a molecular weight of about 90,000 and an isoelectric point of 7.45.[45] On the basis of electrophoresis, ultracentrifugation, and constant solubility, this protein appears to be homomolecular. The hormone stimulates the interstitial tissue of the testis or ovary and, on injection, leads to the formation of corpora lutea provided that maturing follicles are present.

Criteria of Purity of Proteins

It is much easier to name a protein than to characterize it chemically. It is difficult to make certain that a particular protein is pure and homomolecular. Proteins are still often characterized by their mode of preparation rather than by physical chemical criteria. The technique of electrophoresis has demonstrated that some proteins that at one time were considered reasonably pure are mixtures of closely related proteins. The serum globulins are a case in point. With the crystallization of many of the proteins a forward step in the process of purification has been made. However, crystallinity itself is not altogether a criterion of purity. It is necessary at the present time to apply as many criteria as possible to establish the identity and homomolecularity of a particular protein. Such tests as constant solubility at a particular temperature independent of the amount of excess protein added, osmotic pressure in different solvents, diffusion rate, sedimentation constant, electrophoretic mobility, and dielectric constant, together with chemical analysis and crystalline homogeneity, when they are in agreement, can be considered for the present as fairly conclusive evidence of the purity and homomolecularity of a protein. However, the ultimate goal must be that of chemical structure.

Amino Acid Content of Proteins

From a nutritional standpoint † especially it is important that the amino-acid content of proteins be established. The essential and non-essential amino-acid requirements of animals have been determined (a) by inclusion of a single purified protein in the ration, (b) by feeding mixtures of amino acids, (c) by feeding a protein hydrolyzate after chemical removal

* Since this was written Li, C. H., and Evans, H. M., *Science*, **99**, 183 (1944) have purified the growth hormone further and subjected the product to electrophoresis. The protein appeared as a single substance with an I. P. at pH 6.85.

† See Chapter XII for a more extensive discussion of this subject.

of one or more of its constituent amino acids. The deficiency of a protein in one or more amino acids may be represented schematically as follows: In the word

P — R — O — T — E — I — N

let each letter represent an amino acid. Another protein may similarly be represented by the word

R — A — T — I — O — N

If each of the letters in the latter word represents an indispensable amino acid, it is evident that the protein represented by the first word is deficient in that it lacks the essential amino acid represented by the letter *A*. On the other hand it contains the dispensable amino acids represented by the letters *P* and *E*. It should be evident from the above discussion that if for purposes of experimentation an animal is to be maintained on a ration that contains only a single protein, it becomes essential that not only its constituent amino acids be known but also the amount of each amino acid, since it is necessary for purposes of maintaining body weight and permitting growth and reproduction that the animal be supplied quantitative amounts of each of the essential amino acids. Thus since casein has a fairly low methionine content, it must be fed at a level of not less than 18 per cent to rats in order that they may grow. The proteins of the human diet are derived from many sources, and hence it is less likely that a deficiency in a particular amino acid will occur than if only a single protein is included in the diet.

The amino-acid content is a determining factor in the acidic and basic properties of proteins. Thus proteins with a high content of arginine, lysine, and histidine and a low content of aspartic and glutamic acids possess decidedly basic properties. The converse is true unless most of the expected free carboxyl groups of the latter amino acids are present as acid amide, $—CONH_2$, groups. The phenyl ring-containing amino acids appear to influence the antigenicity of proteins. Gelatin, for example, is non-antigenic. With the exception of small amounts of phenylalanine, it lacks the aromatic amino acids. Perhaps gelatin is not a fair example upon which to base such a generalization, since in many respects it differs from native proteins. However, the experiments of Landsteiner [46] have definitely shown that the immunological properties of native proteins may be markedly altered by acylation, methylation, and diazotization. Proteins such as gliadin from wheat and hordein from barley, which have approximately the same amino acid content, interreact to some extent immunologically, indicating that chemical makeup rather than biological origin is the determining factor in immunological specificity. Eventual chemical characterization of proteins will depend on a precise knowledge of their amino-acid content together with information as to the order in which the amino acids are linked.

The amino acid content of many of the naturally occurring proteins is not known and in other cases partial information only is available. Hydrolytic methods have not yet been devised that do not lead to partial or complete destruction of some of the amino acids present in proteins. Methods for the estimation of some of the amino acids are far from precise. Possibly the best data are those that deal with arginine, lysine, and histidine. The above should be borne in mind in interpreting analytic data of proteins. The amino acid content of some of the better characterized proteins is given in Table 1. The figures are given to the nearest decimal. At times even these are without significance due to errors in determination and lack of purity of the protein in question.

Amino Acids and Proteins as Zwitterions. It is now firmly established that amino acids are zwitterions or dipolar ions. The ionization relations are expressed by the equilibria

$$^{+}NH_3 \cdot R \cdot COOH \rightleftharpoons {}^{+}NH_3 \cdot R \cdot COO^- + H^+ \quad \text{or} \quad R^+ \rightleftharpoons R^{\pm} + H^+$$

and

$$H_2O + NH_2 \cdot R \cdot COO^- \rightleftharpoons {}^{+}NH_3 \cdot R \cdot COO^- + OH^- \quad \text{or} \quad R^- \rightleftharpoons R^{\pm} + OH^-$$

The corresponding mass law expressions are

$$K_A = \frac{a_{H^+} a_{R^{\pm}}}{a_R} \qquad \text{and} \qquad K_B = \frac{a_{R^{\pm}} a_{OH^-}}{a_{R^-}}$$

where a denotes activity. In accordance with the above equations, ionization of the carboxyl group takes place during the neutralization of an acid solution of the ampholyte, whereas ionization of the basic group occurs during neutralization of an alkaline solution.

It has become customary to formulate both constants as acidic constants. The ampholyte cation is considered as a dibasic acid:

$$^{+}NH_3 \cdot R \cdot COOH \rightleftharpoons {}^{+}NH_3 \cdot R \cdot COO^- + H^+ \quad \text{or} \quad R^+ \rightleftharpoons R^{\pm} + H^+$$

and

$$^{+}NH_3 \cdot R \cdot COO^- \rightleftharpoons NH_2 \cdot R \cdot COO^- + H^+ \quad \text{or} \quad R^{\pm} \rightleftharpoons R^- + H^+$$

whence

$$K_1 = \frac{a_{H^+} a_{R^{\pm}}}{a_{R^+}} \qquad \text{and} \qquad K_2 = \frac{a_{H^+} a_{R^-}}{a_{R^{\pm}}}$$

When a solution of an ampholyte is placed between two electrodes and a direct current is passed through the solution, the ampholyte ions will migrate to one pole or the other, depending on whether the solution is acid or alkaline. However, at a pH at which the concentrations of anions and cations are equal, no migration occurs. This is the isoelectric point. Expressing the values of K_1, K_2, and I as negative logarithms,

$$pI = \tfrac{1}{2}(pK_1 + pK_2)$$

Table 1. The Amino Acid Content of Some Proteins [1]

Amino Acids (percentage)	Casein	Edestin	Egg Albumin	Fibrin (cattle)	Gelatin	Gliadin	Hemoglobin	Keratin (wool)	Pepsin	Salmin	Serum Albumin	Serum Globulin	Zein
Alanine	1.9	3.6	2.2		8.7	2.0	4.2	4.4			2.7	2.2	9.8
Ammonia	2.3	2.3	1.2		0.4	5.2	1.2	1.4	1.4		1.3		3.6
Arginine	3.8	16.7	5.6	7.7	8.7	2.6	3.6	10.2	1.3	87.4	4.9	5.2	2.0
Aspartic acid	6.0	12.0	8.1	5.9	3.4	1.4	8.9	7.3	6.8		3.1	2.5	1.8
Cystine	0.4	1.4	1.8	1.5	0.2	2.4	1.0	13.1	2.2		5.7	1.1	0.9
Glutamic acid	21.8	20.5	16.1	14.1	5.8	46.9	6.3	15.0	18.6		1.6	8.2	31.3
Glycine	0.5	3.8	0		25.5	0		0.6			0	3.5	0
Histidine	2.5	2.4	1.5	2.5	2.9	3.3	7.6	0.7	0.3		3.4	0.9	0.9
Hydroxyproline	0.2	2.0			14.7								1.0
Isoleucine and Leucine	9.7	21.0	10.7		7.1	6.6	29.0	11.5			20.0	18.7	25.0
Lysine	6.3	2.4	5.1	10.1	5.9	0.7	8.1	2.8	1.7		13.2	6.2	0
Methionine	3.3	2.4	2.4	2.6		2.1		0.7					2.4
Phenylalanine	3.9	3.1	5.1		1.4	2.4	4.2				3.1	3.8	7.6
Proline	8.7	4.1	4.2	5.1	17.5	13.2	2.3	4.4		11.0	1.0	2.8	9.0
Serine	5.8	0.3			3.3	0.1	1.0	2.9		7.8	0.6		1.0
Threonine	4.0				1.4								
Tryptophane	1.2	1.5	1.3	3.0	0	1.1	1.3	1.8	2.2		0.5	2.3	
Tyrosine	6.6	4.6	4.2	6.5	0	3.0	3.2	4.8	10.3		4.8	6.7	5.9
Valine	7.9	6.3	2.5		0	3.4		2.8		4.3			1.9

[1] For further data on this subject, consult Schmidt, C. L. A., "Chemistry of the Amino Acids and Proteins," Charles C. Thomas, Springfield and Baltimore, 1938 and 1944; Cohn, E. J., and Edsall, J. T., "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, 1943.

The content of some of the amino acids in proteins is in need of revision. Thus certain of the tryptophane values are based on a casein standard instead of pure tryptophane. Since β-hydroxyglutamic acid apparently does not occur in proteins, the values for serine and threonine need to be redetermined. Only partial analyses of many of the proteins have so far been carried out. Extensive work in this field is necessary before our knowledge can be considered adequate. For a criticism of some of the analytical methods for the estimation of amino acids, see A. C. Chibnall *et al.*, *Biochem. J.*, **37**, 360, 372 (1943).

If the constants are based on pH measurements, the activity coefficient of the hydrogen ion is included in the value of pI and those of the ampholyte are included in the pK values; hence the values become apparent, and the above equation is written as

$$pI' = \tfrac{1}{2}(pK'_1 + pK'_2)$$

The isoelectric points of the monoamino-monocarboxylic acids are not sharply defined but extend over a zone of several pH units. A narrow zone is obtained only when the values for pK_1 and pK_2 are less than four units apart.

If the amino acid possesses more than two ionizable groups, as in the case of the dicarboxylic amino acids and of arginine, lysine, and histidine, all the ionization constants contribute to the isoelectric point. Using K to denote acidic constants

$$I^2 = K_n K_{n+1} - \frac{1 + \frac{2K_{n+2}}{I} + \frac{3K_{n+2}K_{n+3}}{I^2} + \cdots}{1 + \frac{2I}{K_{n-1}} + \frac{3I^2}{K_{n-1}K_{n-2}} + \cdots}$$

Table 2. Apparent Dissociation Constants and Isoelectric Points of Amino Acids at 25°

	—COOH	—OH	—SH	=NH	—NH_2	pI'
Alanine	2.34				9.87	6.1
Arginine	2.02				9.04	
					12.48	10.8
Aspartic acid	2.09					
	3.87				9.82	3.0
Cysteine (30°)	1.96		8.33*		10.78*	5.1
Cystine (30°)	< 1.0				7.48	
	1.7				9.02	5.6
Diiodotyrosine	2.12	6.48			7.82	4.3
Glutamic acid	2.19					
	4.28				9.66	3.2
Glycine	2.35				9.78	6.1
Histidine	1.77			6.10	9.18	7.6
Hydroxyproline	1.92			9.73		5.8
Isoleucine	2.36				9.68	6.0
Leucine	2.36				9.60	6.0
Lysine	2.18				8.95	
					10.53	9.7
Methionine	2.28				9.21	5.8
Phenylalanine	2.58				9.24	5.9
Proline	2.00			10.60		6.4
Serine	2.21				9.15	5.7
Tryptophane	2.38				9.39	5.9
Tyrosine	2.20	10.07			9.11	5.7
Valine	2.32				9.62	6.0

*The assignment of values to these groups is arbitrary. The pK'_2 and pK'_3 values represent composite constants. The intrinsic acidity of the sulfhydryl group is close to that of the ammonium group. The acidity of each group is strengthened by the presence of the other in the molecule.

For more complete data see Schmidt, C. L. A., "Chemistry of the Amino Acids and Proteins," page 613, 1938 and 1944; Cohn, E. J., and Edsall, J. T., "Proteins, Amino Acids, and Peptides," page 184, New York, Reinhold Publishing Corp., 1943.

In general an aqueous solution of an amino acid is not exactly isoelectric. It will only be so when $K_1K_2 = Kw$. The pH of a dilute solution of any other amino acid without added acid or base will lie between that of its isoelectric point and that of water. As the concentration of the amino acid is increased, the pH of the solution will approach that of the isoelectric point.

Table 2 gives the apparent ionization constants and isoelectric points of most of the accepted amino acids.

Proteins as well as amino acids exist as zwitterions. The isoelectric points of proteins are usually more sharply defined than in the case of simple amino acids. Since the number of acidic and basic groups in a given protein are usually not equal, the isoelectric protein will not have all of its dissociable groups ionized as zwitterions. There will be some un-ionized carboxyl or amino groups, as in the case of a dibasic or diacidic amino acid. As seen from the data in Table 3, the isoelectric points of most native

Table 3. Apparent Isoelectric Points of Some Proteins

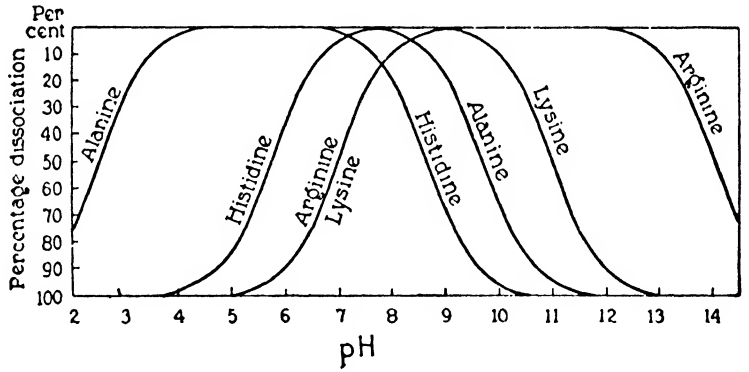
Protein	pI′
Adrenotropic hormone	4.7–4.8
Bence-Jones protein (urine)	5.2
Casein (cow milk)	4.6
Edestin (hemp seed)	5.5–6.0
Egg albumin (hen eggs)	4.55–4.57
Gelatin (calf skin)	4.80–4.85
Gliadin (wheat flour)	6.5
Hemocyanin (snail)	5.1
Hemoglobin, reduced (horse blood)	6.79–6.83
Hemoglobin, oxy- (horse blood)	6.7
Hemoglobin, muscle (horse)	6.99
Insulin (beef pancreas)	5.30–5.35
β-Lactoglobulin (cow milk)	5.20
Lutenizing hormone	7.45
Myosin (rabbit muscle)	6.2–6.4
Ovoverdin	6.7
Prolactin	5.65–5.70
Protamine (fish sperm)	12.0–12.4 *
Serum albumin (horse blood)	4.88
Serum globulin (horse boood)	5.4–5.5
Trypsin (beef pancreas)	5.0–8.0
Urease (jack bean seed)	5.0–5.1

proteins fall below pH 7.0. At the pH of most of the body tissues and fluids in which proteins occur, the proteins exist as anions. Proteins with isoelectric points that lie in the alkaline region are usually combined with nucleic acid or with other prosthetic groups.

At the isoelectric point amino acids and proteins are least soluble; hence it is customary in isolating these substances to adjust the pH of the medium in which they occur to the isoelectric point of the particular product to be isolated. The maximum yields of cystine and tyrosine are obtained by

* The pI′ will vary with different protamines.

adjusting their solutions to their isoelectric points. In preparing casein from milk the pH is adjusted to 4.6. At this pH casein is insoluble and is precipitated. An excellent illustration of the influence of pH is afforded in the separation of certain of the amino acids from a protein hydrolyzate by electrical transport. Foster and Schmidt [47] have shown that when a protein hydrolyzate is placed in the center compartment of a three-compartment cell, the compartments being separated by Cellophane or similar membranes and the end compartments filled with distilled water in which carbon electrodes are immersed, on maintaining the pH of the middle compartment at 5.5 and passing a direct current through the cell, the basic amino acids — arginine, lysine, and histidine — migrate to the cathode compartment, the dicarboxylic amino acids migrate to the anode compartment, while the monoaminomonocarboxylic amino acids remain in the center compartment. On placing the solution of amino acids from the cathode compartment in the middle compartment of another three-compartment cell under conditions similar to those described in the first experiment, except that the pH of the middle compartment is maintained at 7.5, arginine and lysine migrate to the cathode compartment while histidine remains in the center compartment. This is predictable from the dissociation curves of the amino acids.



Percentage dissociation of certain amino acids at varying pH values.

In addition to minimum solubility, proteins show a minimum osmotic pressure, minimum potential difference, minimum swelling (in the case of gelatin), minimum viscosity, and are combined to a minimum extent with acids and bases at the isoelectric point.

Due to their amphoteric nature, proteins can combine with acids and bases. From a stoichiometric standpoint it is important to know which groups participate in these reactions and to what extent. While the available data leave much to be desired, they point to the fact that the acid-combining capacity of proteins can be ascribed to the guanidino group of arginine, the ϵ-amino group of lysine, and the imidazole group of histidine.

The base-combining capacity is due to the free carboxylic groups contributed by aspartic and glutamic acids, the hydroxyphenyl group of tyrosine, plus such acidic groups as may be contributed by phosphoric acid if present. Proteins combine with acid and basic dyes. On the basis of this information Craig and Wilson [48] prepared buffered solutions of stains in order to differentiate tissue structures to a better degree than it is possible when unbuffered stains are used.

From the above limited discussion and the discussions given in the other chapters, it is evident that amino acids and proteins play both a unique and essential rôle in life processes. In order to understand fully their manifold functions, a knowledge of their occurrence, composition, physical chemical properties, and behavior is essential. For more detailed information the reader should consult the larger texts [49] on this subject and the literature cited.

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Chapter III

Protein Structure

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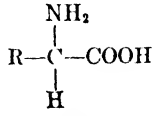
Justus Liebig

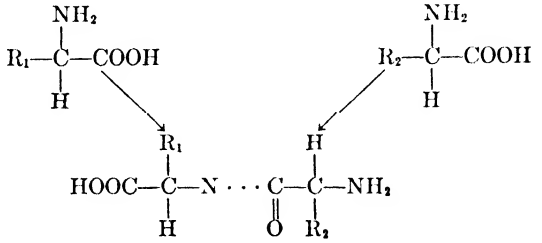
Born in Darmstadt, Germany, 1803, died in 1873. He was the discoverer of chloral, chloroform and tyrosine and founder of the science of agricultural chemistry. He was one of the outstanding organic chemists of his time, and founder of the Liebig *Annalen*.

The amino acids are the building blocks from which proteins are made. A great many amino acids are found in nature, some twenty-two having been identified as constituents of proteins.[1] With the exception of proline and hydroxyproline, they have one important feature in common: they all contain at least one amino group and at least one carboxyl group; and furthermore, these groups bear precisely the same relation to each other, and can be written:



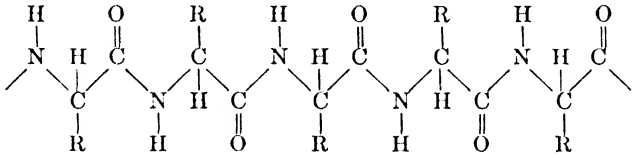
The R-group gives individuality to the amino acid. With the exception of glycine, in which R is a hydrogen atom, R is a hydrocarbon chain to which may be attached a basic or an acidic group; or it may simply be a hydrocarbon chain with no reactive group.

An important chemical property of amino acids is their ability to react with one another to form a peptide bond between the amino group of one amino acid and the carboxyl group of another. In this process a molecule of water is split off.



The dotted line represents the peptide bond.

In the simplest terms, proteins are polymers of amino acids in the same sense as rubber is a polymer of isoprene. The amino acids are linked through the peptide bond to form long peptide chains which may contain several hundred amino-acid residues. There is good evidence that any given protein molecule may be made up of several separate and distinct peptide chains.[2] A segment of a hypothetical peptide chain can be written as follows:



Silk, one of the simplest of the proteins, actually has its peptide chains stretched out — substantially as shown above. Since these chains are all parallel, this fiber has considerable mechanical strength. Apparently all proteins are capable of having their peptide chains stretched provided the proper technique is used. Hair and wool, which are largely composed of the protein keratin, undergo this stretching very easily by simply placing a mechanical load on the hair fiber. Most proteins, however, do not exhibit this transformation so easily and in their natural state have their peptide chains folded in an intricate and as yet unknown fashion.

Note that in the segment of the hypothetical peptide chain given above, the R-groups are free and unattached. These groups determine to a great extent the properties of a protein. If they are mostly acidic, as is the case with the enzyme pepsin, the protein will have pronounced acidic properties. Also important is the polar character of the R groups. Such chemical groups as $—NH_2$, —COOH, $—CONH_2$, OH have a strong attraction for water; these groups are polar or hydrophilic. On the other hand, a hydro-

carbon chain is hydrophobic and is not water-soluble. The balance of the R-groups between polar and non-polar determine to a great extent the water-solubility of a protein.

In addition to the amino acids, which as we have indicated are the main constituent of proteins, a protein may contain a group or groups in which there are no amino-acid residues. Such groups are called prosthetic groups. The classical example of such a group is the heme in hemoglobin. The function of the heme is well understood, but the roles of other prosthetic groups are obscure. In general, a prosthetic group lends stability to a protein. Some proteins, such as insulin, pepsin and trypsin, apparently contain no prosthetic group of any kind.

Molecular Weights. Proteins show a vast range of molecular sizes. Since all proteins have substantially the same density, which is in the neighborhood of 1.34, their molecular weights offer a basis for a comparison of their sizes. The molecular weights have been determined for the most part by the ultracentrifuge,[3] although osmotic-pressure measurements give quite accurate values if carefully done. The ultracentrifuge subjects the protein to a powerful gravitational field and the protein molecules are thrown outward by the centrifugal force. The rate at which they move in the centrifugal field, along with a knowledge of the diffusion constant, allows the molecular weights to be calculated. Such weights range from about 15,000 to several million; there does not seem to be any regularity or system in the distribution of the molecular weights of proteins.

Molecular Weights of Proteins

Protein	M_s	M_e
Ribonuclease	13,000	13,000
Cytochrome c	15,600	—
Myoglobin	16,900	17,500
Bacillus phlei protein	17,000	—
Erythrocruorin (Lampetra)	17,100	19,100
Gliadin	27,500	27,000
Hordein	27,500	—
Crotoxin	30,000	30,500
Oxytocic-press or hormone	30,000	—
Erythrocruorin (Ehironomus)	—	31,500
Human tuberculous bacillus protein	32,000	—
Erythrocruorin (Arca)	—	33,500
Bence-Jones	—	35,000
Pepsin	35,500	39,000
Bence-Jones	37,000	—
Zein	40,000	—
Insulin	41,000	35,000
β-lactoglobulin	41,500	38,000
Concanavalin B	42,000	—
Egg albumin (hen)	44,000	40,500
Hemoglobin (man)	63,000	—
Hemoglobin (horse)	68,000	68,000
Serum albumin (horse)	70,000	68,000
Yellow enzyme	82,000	78,000
Metakentrin	90,000	—

The molecular weights of a number of proteins as determined with the ultracentrifuge are listed on page 75. The molecular weights designated by M_s were found by measuring the rate of sedimentation of the protein molecules in the ultracentrifuge; those indicated by M_e were measured by a study of the distribution of the protein concentration after equilibrium had been reached while centrifuging at a given speed. It is a source of much satisfaction that the molecular sizes of many biologically important proteins are definitely known. The list includes proteins which act as hormones, enzymes, antibodies and respiratory enzymes. Knowledge of the molecular weight is basic to an elucidation of the structural pattern of the proteins.

Molecular Shapes. Very little is known about the actual shapes of protein molecules. It is the custom to classify proteins into fibrous and globular. The fiber proteins are those in which the peptide chains are more or less extended; accordingly, these show enormous asymmetries. Silk, wool, and collagen are examples of fiber proteins. These proteins are as a rule insoluble in water. The globular proteins, while not spherical, do show a low order of asymmetry. These proteins are usually soluble in water, and include egg albumin, insulin, trypsin, pepsin, the serum proteins and numerous others. The only completely unambiguous way in which the shapes of the globular proteins can be measured is by means of x-ray diffraction or by electron-microscopic studies. Both techniques are involved and intricate. So far the resolving power of the electron microscope has not been sufficient to view any but the largest of the protein molecules, and only in the case of tobacco mosaic virus have actual dimensions of the molecule been determined.[4] This protein is rod-like, with considerable asymmetry. Due to technical difficulties x-ray diffraction studies have not been as fruitful in this connection as could be wished. Only in the case of methemoglobin do we have a clean-cut result. According to Boyes-Watson and Perutz,[14] this molecule is a platelet whose dimensions are $36 \times 48 \times 64$ Å.

Both viscosity and diffusion studies are capable of giving approximate information regarding asymmetries. With both types of measurements an assumption has to be made regarding the shape before asymmetries can be calculated. It is customary to express the asymmetries in terms of either a prolate or an oblate ellipsoid of revolution, and it is very improbable that protein molecules have either shape. It is far more probable that they are angular solid bodies which are in the nature of molecular crystals. About all that can be concluded from viscosity and diffusion measurements is that while such proteins as egg albumin, the serum proteins, etc. are probably not cubical, the order of asymmetries of the molecular crystals is not large.

Denaturation. It is agreed by most workers that much can be learned about proteins through a study of protein denaturation. Denaturation is

a characteristic but ill-defined series of changes which many proteins undergo when subjected to a number of relatively mild physical and chemical agents. Among these agents may be mentioned heat, strong acids, and bases, alcohol, alkyl sulfates, urea, high pressures, surface forces, and ultraviolet light, as well as some salts such as lithium thiocyanate. The usual changes in native protein when exposed to denaturing agents are: (1) decreased solubility, (2) increased digestibility by proteolytic enzymes, (3) exposure of oxidizing and reducing groups, notably the sulfhydryl groups, (4) loss of enzymatic properties if the protein is an enzyme, (5) modification of antigenic properties, and (6) decrease of the diffusion constant and increase of the viscosity of the protein solution. Proteins undergoing denaturation do not always show all the changes listed above. There is, however, a definite tendency for these changes to be associated.

While a precise definition of denaturation is lacking, the term does not embrace changes which involve the actual hydrolysis of the peptide linkages.

At the present time the most generally accepted theory of denaturation is that of Wu,[5] who proposed that the change of the native protein to the denatured form could be looked upon as a change from the unique and highly specific structure of the native to the much more random arrangement of the denatured protein. An analogy is the conversion of a crystalline material to an amorphous one. As a broad picture little objection can be raised to this interpretation of protein denaturation.

As we have noted above, if a protein is denatured by some such agent as urea, the viscosity of the protein solution increases. There are two ways of explaining this increase in viscosity. The native protein molecule may be opened up or unfolded to become more asymmetric; an increase in particle asymmetry is known to lead to an increase in viscosity. On the other hand, the protein molecule may have imbibed water and swelled. This likewise would increase the viscosity. Both types of action can be looked upon as an unfolding of the protein molecule from its native condition. The only question involved is whether or not the unfolding gives rise to a more or to a less asymmetric molecule. It is not possible as yet to arrive at an unambiguous decision. The changes in the diffusion constant are always antibatic to those of viscosity, and the same explanations as outlined above for viscosity changes are available to explain the general decrease of the diffusion constant upon denaturation.

Let us consider the viscosity and diffusion of solutions of horse-serum albumin in the presence of urea. Neurath and Saum [6] have studied the change of the viscosity and of the diffusion constant of this protein as a function of the urea concentration. We calculate from their data the volume occupied in solution by the serum albumin on the basis of a spherical molecule by means of the Einstein viscosity equations for spherical particles. We then compare these volumes with those calculated from the

Einstein-Sutherland equation for the diffusion of spherical molecules. Complete agreement would mean that the molecules of serum albumin are actually spherical and the extent of disagreement is a measure of their departure from a sphere. The following table shows the results of such calculations.

Comparison of the Spherical Diffusion Volume with the Spherical Viscosity Volume of Serum Albumin in Urea Solutions

Conc. urea in moles/liter	Ratio of spherical diffusion volume to spherical viscosity volume.
0	0.81
1.5	0.86
3.0	0.89
4.5	0.98
6.0	0.99

The results of these calculations as shown above indicate that the serum albumin approaches a more isotropic shape as the urea concentration is increased, and argue against the idea that denaturation is necessarily accompanied by an increase in a molecular asymmetry.

It has been observed that denatured proteins can be spun into fibers and that in these fibers the peptide chains are more or less extended and no extensive folding is present.[7] In order to achieve this extended configuration, however, some mechanical stress must be applied to the system containing the denatured protein. This does not mean that protein which has been denatured, let us say by urea, and has not been subjected to mechanical stress exists in an extended form. It is clear that if a peptide chain is extended by whatever means may be available, its natural tendency is to fold up again in the same manner that an extended rubber band tends to contract. It is true that if a bundle of peptide chains is present, cross-linking between the chains can hinder or prevent the folding.

It has been noted on several occasions that many denaturing agents tend to produce splitting of some protein molecules. For example, urea splits hemoglobin and myogen into halves, but it does not split egg albumin, pepsin, serum albumin or serum globulin.[8] Those molecules which are split in concentrated urea solution probably possess a plane of cleavage into which urea can penetrate, whereas those molecules which cannot be split by urea involve no such plane of cleavage.

The increased digestibility of a denatured protein means that the peptide bonds are now more accessible to the proteolytic enzyme. This argues for an opening up of the protein structure, whether it be by swelling or by unfolding. The same considerations apply to the exposure of the oxidizing and reducing groups. The change in the biological properties is probably to be accounted for on the basis of the destruction of the surface configuration of the native molecule which can be understood as resulting from either an increase of asymmetry or as a swelling. The decreased solubility of the denatured as contrasted with the native is probably due to the release

of the polar groups which in the native molecule formed intramolecular bonds and which in the denatured state are available for intermolecular bonding.

If a protein is denatured by urea and the urea removed by dialysis it will usually be found that a fraction of the protein has returned to a condition which resembles rather closely that of the native protein as far as its viscosity, solubility, etc. are concerned. The properties of the reversed protein are, however, never identical with those of the native protein. For example, the reversed protein shows a greater degree of digestibility by trypsin than does the native.[9] We can conclude, therefore, that while a denaturing agent such as urea can and does destroy the specific configuration which is characteristic of the native protein, upon removal of the urea, it is possible for the peptide chains to return to a type of fold which resembles that of the native.

By a study of the rate of denaturation as a function of the hydrogen-ion concentration it has been possible to conclude that the stability of the native protein molecule depends in large measure on the interaction of charged groups in the molecule.[10] These charged groups are for the most part amino and carboxyl groups in the R-groups. Recent studies on the ultraviolet adsorption spectrum also indicate that in the native protein the tyrosine residues containing the phenolic OH group are bound, and are liberated only in rather high alkali concentration. These groups along with other groups are capable of forming hydrogen bonds. Hydrogen bonds are weak chemical bonds acting through a somewhat longer distance than do ordinary chemical bonds. They arise when oxygen or nitrogen atoms share a common hydrogen atom, the hydrogen atom serving to bond the two electronegative atoms. Hydrogen bonds probably play an important role in holding the peptide chains in a given configuration, and these bonds along with the purely electrostatic interaction mentioned above, are probably crucial for the existence of the specific configuration of the native molecule.

To summarize the information about proteins which has been obtained from denaturation studies, we can say that the native molecules exist in a highly ordered arrangement. This order probably resides in the specific folding and fitting together of peptide chains in such a manner as to yield a molecular crystal.

Spread Monolayers. Before leaving the question of protein denaturation we must consider surface denaturation. If a drop of dilute protein solution is placed on a clean water surface, the protein will spread over the surface and completely cover it, and if the experiment has been properly done, all the protein will remain in the surface and none of it will go into solution. This surface film can be compressed by moving a glass or metal strip along the surface. The film can be compressed against a mica float connected to a torsion balance and the film pressure measured as a function of the area

of the film. We can then plot the film pressure against the film area. If we do this we find that as the film is compressed, its compressibility decreases until a critical pressure is reached, at which point the compressibility sharply increases. It is considered that at this point of minimum compressibility the protein film is in its most compact state and that pressures above this cause the film to collapse. From the weight of protein placed on the surface and the area of the film at the point of minimum compressibility the area of the surface per given weight of protein can be calculated. Also, since we have a rough idea of the density of the protein in the film, the film thickness can be calculated. Most protein films exhibit remarkable similarity in dimensions. They all occupy in a compressed state from 0.8 to 0.9 square meter per milligram of protein and are from 9 to 10 A thick.

In order to spread on a water surface, a substance must contain exposed polar and non-polar groups. The polar groups are water-soluble, and bring about spreading, whereas the non-polar groups (hydrocarbon chains) prevent the substance from passing into solution. As we have noted, proteins contain hydrophobic as well as hydrophilic residues. In a spread film the hydrophilic groups are oriented into the water phase, the hydrophobic groups pointing upward. The lower surface of the film is thus hydrophilic and wet by water, while the surface exposed to the air is hydrophobic or "oily."

One dramatic aspect of surface films of proteins is the speed with which they form. If the spreading has been done on concentrated salt solutions, the film can be compressed immediately after spreading and it will occupy the same area at the point of minimum compressibility as it does if it is compressed at the end of two hours. In view of the readiness with which proteins spread and form a surface layer it seems most probable that these layers must pre-exist in the native molecule. In short, the spreading of the native molecule involves the unleafing of layers of peptide chains in the molecule.

Both egg albumin and β-lactoglobulin form gaseous films on 35 per cent $(NH_4)_2SO_4$ solutions at film pressures below 0.5 dyne. By the application of the gas laws in two dimensions it is possible to calculate the molecular weight of these two proteins in the surface films. It turns out that their molecular weight in the surface film is the same as in bulk solutions, and it can therefore be concluded that neither protein undergoes dissociation or association on the surface. It is also possible to estimate the area of the gaseous molecules, which is found to be not very much greater than for the most highly compressed but uncollapsed film. This indicates that there is extensive orientation of the side-chain residues and that the peptide chains do not spread apart to any great extent.

After a protein film is spread it is possible to deposit the film on a properly treated glass or metal slide by slowly raising the slide up through the surface on which the film has been spread. It is found that such films

retain their power to combine with a specific antibody,[12] and if they are composed of enzymes, the surface films retain their enzymatic properties.[13] If, as we believe, these biological properties are associated with the specific folding of the peptide chains and with the resulting mosaic pattern on the surface of the native molecule, then we conclude that the spread film retains the specific folds of the peptide chain that it had in the native state. This conclusion is in agreement with the hypothesis that the surface films of protein must pre-exist in the native molecule as leaflets.

Molecular Structure. Significant for this discussion are the results of x-ray diffraction studies of Boyes-Watson, and Perutz [14] on horse methemoglobin crystals. These workers determined the x-ray spacings of crystals of methemoglobin in the dry and in the wet condition. They conclude, as we have noted before, that the methemoglobin molecules are platelets which are about 36 Å thick, 64 Å long, and 48 Å wide. In the crystal, the platelet molecules are linked with their neighbors to form coherent layers which remain parallel at all stages of shrinkage as drying proceeds. The water of crystallization is distributed in sheets between layers of protein molecules. The water of crystallization is thus entirely intermolecular. These investigators further conclude that the hemoglobin molecule is made up of four parallel layers of scattering matter which are a little less than 9 Å apart. These layers are not, however, penetrated by water. A layer structure for the globular proteins has also been suggested by Astbury,[15] by Pauling,[16] by Dervichean,[17] and by Palmer.[18]

As we have noted, some proteins such as hemoglobin split into halves in concentrated urea. Such protein molecules probably have a hydrophilic plane of cleavage in the molecule into which urea can enter, with consequent splitting of the molecule. It is not difficult to picture in a general way the main features of the hemoglobin molecule. Since hemoglobin spreads in monolayers on clean surfaces of buffer solutions, it is probable that the four layers of peptide chains which are indicated by the work of Boyes-Watson and Perutz have both hydrophilic and hydrophobic faces. In the hydrophilic faces are included the hydrophilic side chains of the amino-acid residues, while the hydrophobic faces include for the most part the hydrophobic side-chain residues. The hydrophilic face of the top layer of peptide chains is directed upward; since hemoglobin is water-soluble, the outer surface of the molecule must be hydrophilic. Below the first layer of peptide chains is a second layer, in which the hydrophobic residues are directed upward to yield a hydrophobic plane between the first and second layers of peptide chains. The second layer of peptide chains has its hydrophilic face directed downward and is in contact with the hydrophilic face of the third layer of peptide chains. This gives rise to a hydrophilic plane in the center of the molecule into which urea can penetrate and along which is the plane of cleavage of the molecule. In contact with the hydrophobic face of the third layer is the hydrophobic face of the fourth layer. The

bottom face of the molecule is again hydrophilic. The general picture of the molecule is thus two hydrophobic sandwiches pressed together and capable of being divided along the mid-hydrophilic plane.

There is some reason to believe, however, that such proteins as egg albumin, pepsin and β-lactoglobulin are not four-layer structures. Such molecules do not split in concentrated urea solutions. The writer believes that a two-layer structure for these proteins is in keeping with everything we know about them; and since this represents a simpler assumption than a four-layer structure, a structure consisting of two parallel layers of peptide chains will be tentatively accepted for these proteins. In such a structure the outer faces would be hydrophilic and would contain all or practically all the charged groups The inner planes would contain for the most part the hydrophobic groups. Spreading on the surface would involve the opening of the molecule in the same manner that a book is opened. This opening, at least in the cases of egg albumin and β-lactoglobulin, does not involve dissociation of the molecules.

The thickness of all two-layer structures would be about 19 A; and since all or practically all the acidic and basic groups of these proteins can be readily titrated with acids and bases, these groups must be exposed and located in the outer faces of the molecule. Dielectric-constant studies indicate a very uniform distribution of these charged groups. Upon the addition of acid, these groups assume a net positive electrostatic charge. This net charge would tend to cause a horizontal expansion of the two layers with a resulting instability of the molecule. The same effect should be observed on the alkaline side of the isoelectric point and would be due to the accumulation of a net negative charge.

Urea would be expected to penetrate between the peptide chains and accordingly would bring about an expansion of the peptide layers. Further action would lead to a disruption of the layers with a more or less random arrangement of the peptide chains. We have indicated our reasons for believing that in very concentrated urea solutions, serum albumin molecules tend to assume a shape which approaches a spherical condition. The alkyl-sulfated detergents probably penetrate into the peptide layers of the native molecule in a manner analogous to the penetration of spread monolayers of protein by surface-active agents, as described by Schulman and Rideal.[19] The hydrophobic tails of the alkyl sulfate molecules would be located in the hydrophobic part of the peptide layer, while their sulfated heads are attached to the positively charged groups of the hydrophilic layer.

Little can be said about the actual arrangement of the peptide chains in the layers. Close packing of the residues is no doubt present, and the arrangement and folds are certainly critical functions of the nature of the amino-acid side-chain residues as well as the locations of these residues in the peptide chains.

The theory of protein structure discussed in this paper is still tentative and any one who has watched the passing parade of theories of protein structure and who has listened to the barkers crying the virtues of their wares will regard the whole affair with the cold eye of skepticism. Such an attitude is a sound one. A layer structure for proteins is however in accord with too many of our experiences to be lightly dismissed; and while future work will no doubt result in modification and extensive elaboration, such a structure has the ring of reality.

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Chapter IV

Hydrolysis of Proteins

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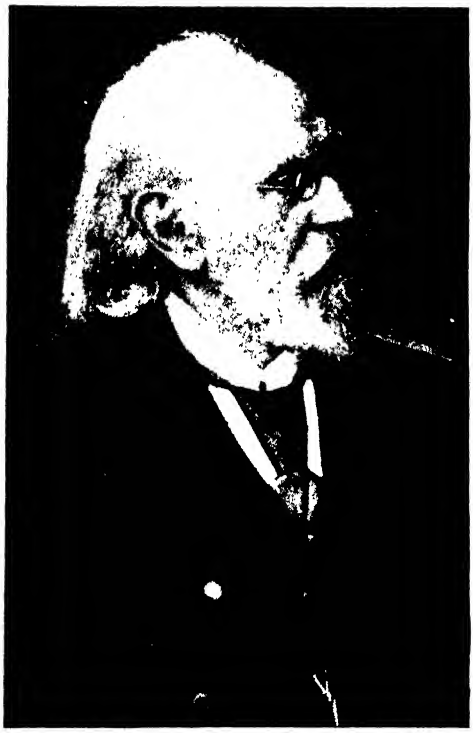

Emil Erlenmeyer, Sr.

Born in 1825, at Welmen near Wiesbaden Germany, and died in 1909 His early research was on the constitution of amino acids. He synthesized tyrosine and established its chemical structure. He also synthesized leucine. For many years he was editor of Liebig's *Annalen*.

Historical

Our understanding of the chemical structure of the proteins and their components, the amino acids, has depended to a large extent on methods of hydrolysis. In 1819, Proust [1] discovered leucine while investigating the fermentation of gluten and milk curd. In 1820, Braconnot [2] employed sulfuric acid as an agent for the breakdown of gelatin and obtained glycine. This is probably the first report of the use of sulfuric acid for the hydrolysis of proteins. In 1839, Mulder,[3, 4] who was not enthusiastic over the use of acids as hydrolytic agents, particularly hydrochloric acid, introduced the method of hydrolyzing proteins by an alkali. In 1846, Liebig [5] fused casein

with potassium hydroxide and discovered tyrosine. In 1849, Bopp [6] introduced hydrochloric acid and demonstrated successfully its usefulness for the hydrolysis of proteins. He obtained tyrosine from casein. In 1848, Piria [7] studied the effect of nitrous acid on asparagine and aspartic acid and converted them into malic acid with loss of nitrogen. This marks the discovery of deamination. In 1873, Hlasiwetz and Habermann [8] introduced the novel method of hydrolyzing proteins with hydrochloric acid in the presence of stannous chloride. They found that the use of this reducing agent minimizes the formation of humin. Titanous chloride [21] and zinc dust or zinc chloride [22] have been used for the same purpose.

It is therefore obvious that we can obtain amino acids from proteins by the following hydrolytic methods: (1) by acids, (2) by alkalies, and (3) by enzymes. Each of these methods has its advantages and disadvantages. There is as yet no perfect method for hydrolyzing proteins.

General Considerations

The treatment of a protein with an acid at ordinary room temperature is not sufficient to bring about complete breakdown of the protein molecule into its components. The following rules for hydrolysis by an acid or by an alkali must be observed.

Concentration of Acids and Alkalies. In most instances in the literature, the amounts of acids and of alkalies used have been reported in percentages. This is indeed unfortunate, for it is obvious that acids and alkalies contain certain amounts of impurities as well as variable amounts of water. Thus, the exact concentration of sulfuric acid or hydrochloric acid used to hydrolyze proteins by Braconnot,[2] Bopp,[6] Hlasiwetz and Habermann,[8] and by more recent investigators was probably not identical with the concentrations we are now using in our laboratories. Furthermore, even in one's own laboratory, when a bottle of concentrated sulfuric acid is once opened and not properly stoppered, the acid will take up a certain amount of water, or when a bottle of concentrated hydrochloric acid is once opened, the concentration of acid does not remain the same. Also some hydrochloric acid is lost during boiling. Alkalies, such as potassium hydroxide and sodium hydroxide, absorb carbon dioxide as well as a considerable amount of moisture. Therefore, if one wishes to perform accurate hydrolysis and be in a position to duplicate his own results, it is necessary to express the concentration of acids and alkalies in terms of normality.

The choice of an acid or an alkali depends considerably on the nature of the protein, and on the particular amino acid that an investigator wishes to isolate. As an example, for the preparation of cystine the use of an alkali or of hydrochloric acid with stannous chloride is not suitable for the hydrolysis of hair or wool. The hydroxide destroys cystine and the presence of stannous chloride in hydrochloric acid reduces cystine to cysteine. On the other hand, if one desires to prepare diiodotyrosine or thyroxine, he should

employ an alkali, such as barium hydroxide, as a catalytic agent. (See Chapter I.)

Concentration of Proteins for Hydrolysis. Whether we select an acid or an alkali as a hydrolytic agent, it is desirable to specify not only the amount of acids or of hydroxides but that of the proteins in the volume of the final mixture. The conditions that govern the rate of hydrolysis of proteins must be in accord with the Law of Mass Action. The law states that *the velocity of a reaction at constant temperature and pressure is proportional to the product of the concentration of the reacting substances, the concentration being expressed in gram molecules per liter.* It is, therefore, obvious that for constant and accurate results, the concentration of the catalytic agent as well as that of the substrate must be known. For example, a mixture containing one gram of casein in 100 cc 5*N* sulfuric acid can be hydrolyzed by boiling in much shorter time than a mixture containing 10 grams of casein in 100 cc 5*N* sulfuric acid under the same conditions. It is advisable to avoid boiling of proteins in acids or in alkalies beyond the time required for the completion of hydrolysis, as some amino acids may be partially destroyed or racemized.

Rate of Hydrolysis. Dunn [23] investigated the rate of hydrolysis of casein by acids and on the basis of a single series of measurements reported that it fitted a first-order reaction. Greenberg and Burk [24] studied the hydrolysis of gelatin, gliadin, and silk fibroin by different concentrations of hydrochloric acid and sulfuric acid. Their data indicated that the rate of hydrolysis of these proteins as measured by the increase in amino nitrogen was that of a second-order reaction. They also showed that the catalytic action of acids on protein hydrolysis is proportional to the thermodynamic activity of the hydrogen ion. Nasset and Greenberg [25] reinvestigated the acid hydrolysis of casein and found that its rate conformed to the equation for a second-order reaction:

$$K = \frac{1}{t} \times \frac{x}{100 - x} \times \frac{1}{100}$$

in which x represents the percentage of protein hydrolyzed in time t.

Effect of Temperature and Pressure. Obviously the higher the temperature, the faster is the rate of hydrolysis. The general procedure that has been handed down to us has been to boil and reflux the mixture containing the catalytic agent and the protein until the reaction is completed. The rate of hydrolysis can be speeded up if the same mixture is heated under pressure. Again, it is preferable not to continue hydrolysis beyond the time required for its completion.

Hydrolysis of proteins by acids or by alkalies under steam pressure in an autoclave has decided advantages over hydrolysis by boiling and refluxing. Greenberg and Burk [24] studied the effect of temperature on the rate of acid hydrolysis of gelatin and demonstrated that the velocity constant is a function of temperature and can be expressed by the following equation:

$$\text{Log } K_a = 0.0287\ T - 5.30$$

in which K_a refers to the velocity constant at unit activity and T is expressed in degrees centigrade.

It is therefore obvious there is considerable economy in time and material when we hydrolyze proteins by acids or alkalies under steam pressure and at an elevated temperature.

Completion of Hydrolysis. Hydrolysis of a protein is judged complete when the peptide linkages are entirely broken. This is rather difficult to determine accurately. The biuret test, which is indeed useful and extensively employed for the purpose of detecting peptide linkages, is not conclusive. The following amino acids give positive biuret tests: histidine, serine, and threonine. Thus, it can be seen that a positive biuret reaction is not specific for a peptide linkage. The best method recommended for determining the completion of hydrolysis of a protein, is the estimation of total α-amino-nitrogen and total nitrogen and the establishment of the ratio: $\frac{\alpha\text{-Amino-N}}{\text{Total N}}$. If the constituents of a protein are free from any of the following amino acids, arginine, histidine, lysine, tryptophane and cystine, then on digestion its total nitrogen is equivalent to total α-amino-nitrogen and the ratio is 100.* In view of the common occurrence of most of these amino acids in proteins, values for α-amino-nitrogen are constantly lower than those for total nitrogen, and the ratio is less than 100. It therefore becomes desirable to establish a definite ratio for each hydrolyzate of a particular protein.

Humin. The consensus of opinion concerning the origin of humin is that it is formed by the condensation of tryptophane with an aldehyde. Gortner and his co-workers [15] pointed out that most proteins contain either an aldehyde group or some other group that reacts like an aldehyde. On the other hand, it is difficult to obtain carbohydrate-free proteins. There is also a possibility that tyrosine in the presence of traces of metals, such as iron, may also give rise to the formation of humin. It is believed that melanin is obtained from tyrosine by the action of tyrosinase. The hypothesis that the amount of tryptophane in a protein is proportional to the humin formed by the acid hydrolysis awaits confirmation.

Ammonia. When a protein is completely digested by an acid and subsequently made alkaline, ammonia is liberated and can be quantitatively recovered by distillation. For its estimation it is advisable to use mild alkalies such as calcium oxide or magnesium oxide. The source of this ammonia is believed to be the acid amide — $CONH_2$ groups of the dicarboxylic acids, aspartic and glutamic. In many instances it is referred to as amide nitrogen. Gortner and Holm [16] reported that all of the amide nitrogen in a 24–48-hour hydrolysis was not derived entirely from acid amide linkage. These investigators were of the opinion that some ammonia resulted from deamination of the amino acids. They based their theory on

* According to Dunn [26] glycine and cystine yield more than 100 per cent amino nitrogen.

the results they obtained following the treatment of an ammonia-free hydrolyzate of protein with 20 per cent hydrochloric acid, and boiling the mixture for 13 days. They reported a considerable amount of ammonia.

I. Hydrolysis by Acids. The most common catalytic agents are sulfuric acid and hydrochloric acid. Other acids have also been investigated. Baernstein [27] hydrolyzed proteins with 57 per cent hydriodic acid in the presence of dihydrogen phosphate. Miller and du Vigneaud [28] employed a mixture of hydrochloric acid and formic acid for the hydrolysis of insulin protein. Sullivan and Hess [21] and subsequently Hess and Sullivan [29] used titanous chloride in the presence of hydrochloric acid and reported that this mixture hydrolyzes proteins with the formation of little humin and with a shortening of the time of hydrolysis.

Phosphoric acid, acetic acid, and acetic anhydride are not sufficiently strong acids to break the molecule of protein into its constituents. Lactic acid and gluconic acid were investigated by the writer. They are not suitable.

Sulfuric Acid. This was the first acid used for the hydrolysis of proteins. Its advantages were recognized by the early investigators of protein chemistry, for the simple reason that its anions can be almost entirely removed by the cations of calcium oxide or of barium hydroxide. Its main drawback is that the voluminous precipitates of calcium sulfate or of barium sulfate retain or adsorb a certain amount of the constituents of the digested proteins. Thus, for accurate quantitative estimation of the individual amino acids, it may be advisable to use hydrochloric acid.

Hydrochloric Acid. On account of the difficulties encountered in the removal of its anions, this acid was not widely used until the early part of this century, when Fischer [11, 13] introduced his method of esterification of amino acids. It is a very efficient catalytic agent and is preferred to sulfuric acid by numerous investigators.

Certain Disadvantages of Acid Hydrolysis. Hydrolysis of proteins by sulfuric acid or by hydrochloric acid leads to the destruction of tryptophane and the possible dismutation of a certain amount of cystine, serine, threonine, and the dicarboxylic acids.[14] With the exception of zein and pepsin, most proteins, when boiled with either of these strong catalytic agents, yield a black or brownish-black substance termed melanin or humin.

Example. The following is an example for the hydrolysis of casein by sulfuric acid under pressure.[22]

Into a 20-liter Pyrex bottle containing 14 liters of 5*N* sulfuric acid introduce 2 kg of casein and mix intimately. Cover the bottle with a piece of cheesecloth and place it in an autoclave, gradually increasing steam until the pressure reaches 15 pounds per square inch and an inside temperature of about 120° C. Autoclave for 16 consecutive hours, then gradually release the steam pressure, remove the acid hydrolyzate and cool. Add calcium oxide (slaked lime) until the reaction is approximately pH 10.0. Adjust the volume of the alkaline mixture to about 30 liters by the addition of dis-

tilled water. Stir thoroughly and filter. Return the calcium sulfate precipitate to the original container and add to it about 10–12 liters of hot distilled water; stir for about 30 minutes and filter. Combine the filtrates and concentrate *in vacuo* to a volume of about 10 liters. Saturate the ammonia-free filtrate with carbon dioxide and cool in a refrigerator. Most of the calcium is removed as calcium carbonate. Filter and add enough oxalic acid to precipitate the remaining calcium ions. To avoid the presence of oxalic acid, it is preferable to have a slight excess of calcium ions. Heat the filtrate to about 60° and carefully add just enough barium hydroxide to remove all but a trace of sulfate. Filter while hot. Determine the total nitrogen and α-amino-nitrogen and concentrate the hydrolyzate, *in vacuo*, to the desired volume, so that total nitrogen is about 2.1 per cent. (This represents a concentration of 15 per cent amino acids.) The hydrolyzates are tryptophane-free and have a ratio of about 82 for $\frac{\alpha\text{-Amino N}}{\text{Total N}}$. For parenteral administration, add one per cent tryptophane in proportion to the amino acids present. Filter through a clarifying pad and sterilize by passing the mixture of amino acids through a sterile Berkefeld filter.

II. Hydrolysis by Alkalies. Hydrolysis of proteins by such catalytic agents as sodium hydroxide, potassium hydroxide, and barium hydroxide is limited in its scope, despite the fact that proteins are completely hydrolyzed to their constituents. Recently Steinhardt and Fugitt [18] reported on the use of certain wetting agents, the sulfonate half-esters of organic acids, as hydrolytic agents for proteins. This is indeed a new method of approach and merits further consideration by investigators in this field. However, these reagents do not appear to hydrolyze the protein completely.

Barium hydroxide has been widely used for the isolation of thyroxine from thyreoglobulin and for the preparation of iodogorgoic acid from sponges and the skeletons of certain corals. The main advantage that hydroxides have over acids is that they do not cause the formation of humin. Thus, according to reports, they do not destroy tryptophane. Thyroxine and iodogorgoic acid are not affected by their catalytic action. On the other hand, they cause complete or partial decomposition of cystine, arginine, and lysine.

When a protein is hydrolyzed by an alkali, its constituents, with the exception of glycine, are racemized. Those interested in preparing amino acids from proteins for the purpose of feeding experiments should realize that not all the racemic amino acids are utilized by the animal. A discussion on the utilization of racemized amino acids is found in Chapters IX and X on metabolism and in Chapter XII on the rôle of amino acids in nutrition.

III. Hydrolysis by Enzymes. Hydrolysis of proteins by enzymes cannot be adequately covered in this review. We can only present a brief outline. Those who are interested in pursuing this subject at greater length should

consult treatises on enzymes, some of which are listed in the bibliography at the end of this chapter.

Those enzymes that are capable of catalyzing the hydrolysis of proteins are termed proteolytic enzymes. They are numerous and abundant in nature. Some are obtained from animal organs and some are of vegetable origin. We have already learned in Chapter II and Chapter III that proteins consist of large molecules. When these molecules are attacked by acids, by alkalies and by enzymes, they are not decomposed directly to simple amino acids. Their degradation products follow a definite pattern: Protein ⟶ Proteoses ⟶ Peptones ⟶ Peptides ⟶ Amino Acids. Some amino acids are liberated much sooner than others.

Schulze and co-workers were the first to study the action of vegetable enzymes on proteins. Subsequently, Kühne, Chittenden, Kossel, Kutscher, Drechsel, Willstätter, Waldschmidt-Leitz, Northrop, Sumner and many others contributed largely to our knowledge of the chemistry, behavior, preparation and mode of action of these complicated substances. It has been the consensus of opinion that *in vitro* the hydrolysis of proteins by enzymes is never complete. Dunn and Lewis [19] studied the action of pepsin, trypsin and erepsin on casein and reported that after prolonged digestion they obtained almost a complete degradation of protein to amino acids. The writer reinvestigated this problem and was able to substantiate Dunn and Lewis's findings. A brief summary of Sahyun's unpublished data on the hydrolysis of casein by trypsin, then by erepsin, and finally by erepsin and activators is as follows:

	$\frac{\alpha\text{-Amino N}}{\text{Total N}}$	Hydrolysis (Per cent)
Tryptic digestion of casein	47–52	57.5–63.5
Ereptic hydrolysis of tryptic digest	71–72	86.5–87.5
Ereptic hydrolysis of tryptic digest plus activators	75–76	91.7–92.0
Completion of hydrolysis with acids	82	100.0

Data for per cent hydrolysis are calculated by dividing the ratio of the enzymic hydrolyzate by that of the acid hydrolyzate.

In one experiment, however, the writer found that the α-amino N of one digest approximated that of an acid hydrolyzate, but unfortunately, the substrate was found to be contaminated with microörganisms and there was unmistakable evidence of putrefaction.

Enzymes that are capable of catalyzing the hydrolysis and the degradation products of proteins have, for many years, been classified according to the degree and complexity of their substrates. Those that can attack the high-molecular proteins are pepsin, trypsin, cathepsin, papain, etc. They are termed *proteinases*. Those capable of splitting the degradation products of proteins, such as peptides, to still smaller fragments are called *peptidases*. Thus, there are dipeptidases, tripeptidases, and polypeptidases, and these as a rule hydrolyze peptides to simple amino acids.

Hydrolysis of proteins by enzymes must be carried out under specific conditions. An intimate knowledge must be acquired of the potency of the enzyme, its activation and inactivation, its specificity, the acidity of its optimum catalytic effect, and the temperature.

The optimum pH for the activity of certain proteolytic enzymes is worth noting. Pepsin acts best at pH 1.5 and hydrolyzes proteins to proteoses and peptones. Pancreatic trypsin requires an alkalinity of pH 7.8–8.0 and slowly catalyzes the hydrolysis of some proteins, preferably if denatured by heat, and more readily proteoses and peptones to peptides and amino acids. Papain, which is derived from the latex of *Carica papaya*, hydrolyzes most proteins, proteoses, and peptones to peptides and amino acids and requires a pH of about 7.0.[30] Erepsin, which at one time was considered a single enzyme, is now known to consist of several peptidases that can catalyze the hydrolysis of peptides to simple amino acids at about pH 7.0. In the opinion of the writer, the optimum activity of erepsin is at about pH 7.5.

The mild conditions of acidity and alkalinity and of temperature under which proteolytic hydrolysis proceeds is of considerable advantage in investigations of the amino acids content of proteins. This is of particular interest when it is desired to obtain the natural unracemized amino acids without subjecting them to the drastic action of strong acids and alkalies.

*Example.** *Tryptic Digestion.* Into a suitable vessel containing about 700 cc of distilled water and 10 cc of chloroform, introduce 100 grams of casein, stir and add enough 5*N* sodium hydroxide to make the reaction definitely alkaline to phenolphthalein. Then add 0.5 gram of active pancreatin (potency 1:300) suspended in about 100 cc of water and continue stirring for an hour. Readjust the alkalinity of the mixture to pH 8.0 by careful addition of sodium hydroxide. Bring the volume of the mixture to 1000 cc. Mix intimately and place the contents in an Erlenmeyer or Florence flask. Add enough toluene (25–30 cc) so that a thin layer of the preservative remains on the surface after shaking. Stopper the container (cork is preferable to rubber) and incubate at about 37° C. On the following day determine the alkalinity of the mixture by means of a glass electrode and adjust to pH 7.8 to 7.9 by the addition of more sodium hydroxide. Shake well at least once a day without opening the flask. After 4 days, add another 500 mg of pancreatin (potency 1:300) and check the alkalinity. It should be about pH 7.8. Mix well. If most of the toluene has evaporated, add another 10–15 cc to insure the presence of a thin layer over the surface. Incubate for another 4 days. After an overall incubation of 8 days, determine the α-amino nitrogen and total nitrogen. The ratio should be about 48, provided active pancreatin is used and the hydrolysis is complete. Further addition of trypsin and incubation has little, if any, effect on digestion.

* From unpublished data by Melville Sahyun.

Ereptic Digestion. Adjust the acidity of the tryptic digest to pH 7.6 and add 5–10 grams of defatted, minced, fresh, small intestines, selecting the first 6–8 inches of the jejunum. (This organ should be rapidly washed with cold distilled water prior to mincing). If the addition of an activator is desired, 2 cc of 0.10*M* manganese sulfate per 100 cc should be added to the mixture. Incubate for 2 or 3 days. Determine the α-amino nitrogen and total nitrogen. When a ratio of about 75 is obtained, further addition of erepsin has no effect.

For the preparation of a sample of hydrolyzate for the modified formol titration, the following procedure was found suitable: Remove a sample of 50 cc and acidify with sulfuric acid to about pH 3.5. Add enough water to bring the volume exactly to 100 cc. Mix well and heat the mixture to about 50–60° C. Insoluble matters, such as undigested casein, flocculate and the clear filtrate of the hydrolyzate is obtained. Determine the α-amino nitrogen and total nitrogen and multiply the result by two to correct for dilution.

Hydrolysis of Casein by Enzymes *

Time (days)	Enzymes	$\frac{\alpha\text{-Amino N}}{\text{Total N}}$	Hydrolysis
	Trypsin	Ratio	Per cent
1	"	32.9	40.0
2	"	38.8	47.5
5	"	45.0	55.0
6	"	45.7	56.0
7	"	47.5	58.0
8	"	48.4	58.9
The tryptic digest was acidified, filtered, adjusted to pH 7.6 and diluted so that 1 cc contains 5 mg N, and analyzed.			
8	Trypsin	48.6	59.3
9	"	50.5	61.5
Erepsin was added and samples incubated for 2 days.			
11	Erepsin	71.0	86.5
12	"	71.5	87.0
After the 12th day 2 cc of 0.1*N* manganese sulfate was added.			
13		75.5	92.3
14	No further change.		
14 Control. No manganese sulfate added.		72.0	87.7
Hydrolysis of enzymic digest with sulfuric acid.		82.0	100.0

Formol Titration. α-Amino nitrogen can be accurately determined by the modified formol titration method of Sörensen. This method has been used by the writer since 1935, and is similar to Dunn and Loshakoff's method.[20] Introduce 10 cc of the ammonia-free casein digest into a suitable titration flask and adjust the acidity to exactly pH 6.0. At this pH the digest has practically no buffer effect. Therefore, care must be exercised in the addition of acid or alkali. Add 10 cc of 37.5 per cent formaldehyde (C.P.) and 0.5 cc of phenolphthalein. The addition of the indicator is merely used as a guide. From a calibrated burette titrate against 0.2*N*

* From unpublished data by Melville Sahyun.

sodium hydroxide exactly to pH 9.0 (using a glass electrode for pH determination). Measure the exact amount of 0.2*N* sodium hydroxide added and subtract from it the equivalent of the standard alkali required to titrate 10 cc of formaldehyde in water (control). Multiply the difference by 2.8. The result represents the amount of α-amino nitrogen in the sample.

Example. Assuming that 10 cc of formaldehyde required 0.6 cc of 0.2 normal sodium hydroxide and 10 cc of the hydrolyzate required 25.6 cc of 0.2*N* sodium hydroxide, then 25.6 − 0.6 = 25 cc 0.2*N* NaOH. 25 cc × 2.8 = 70 mg α-amino nitrogen per sample. Since 10 cc of hydrolyzate was used, then there are $\frac{70}{10}$ = 7 mg α-amino nitrogen per 1 cc.

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Chapter V

Synthesis and Isolation of Certain Amino Acids

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Adolph Friedrich Ludwig Strecker

Born in Darmstadt, Germany, in 1822 and died in 1871. He was an assistant to Liebig and discoverer of the well-known Strecker cyanohydrin synthesis. A careful and painstaking investigator.

SYNTHESIS OF AMINO ACIDS

A wide variety of general and special methods for preparing amino acids has been developed during the past hundred years. The object of this section will be, not to cover the literature in detail, but rather to outline the useful methods, indicating the principles involved, and pointing out the advantages and disadvantages, and any unusual features of each of the reactions. No attempt will be made to list all the amino acids prepared by any particular method. However, the important applications of each will be listed and the best methods for preparing each of the naturally occurring amino acids will be summarized at the end of the section. For further detail the reader is referred to the excellent reviews on synthesis and reactions of amino acids which have appeared in the last five years.[1,2,3] Excellent lab-

oratory directions for the preparation of many of the amino acids are given in *Organic Syntheses*.

I. Amination of α-Halogen Acids

$$R{-}\underset{\displaystyle X}{\underset{|}{CH}}{-}CO_2H + NH_3 \longrightarrow R{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}CO_2H + NH_4 \cdot X$$

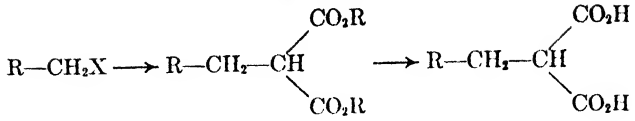
One of the oldest and most general methods of preparing amino acids consists of treating an α-halogen acid with ammonia. α-Bromo acids are most often used since they are readily prepared and are more reactive than α-chloro acids. Furthermore, ammonium bromide is separated from amino acids more readily than is ammonium chloride. A wide variety of conditions has been employed for the amination. The ammonia is usually supplied in the form of concentrated ammonium hydroxide, although ammonia in alcohol has also been used. Cheronis and Spitzmueller [4] reported that the addition of ammonium carbonate to the ammonium hydroxide generally improved the yield. In any case a large excess (10–15 moles) of ammonia is used in order to decrease side reactions such as formation of a secondary amine and hydrolysis of the bromo acid to an α-hydroxy acid. The reaction conditions vary from standing at room temperature to heating in a bomb at 100° C or above.

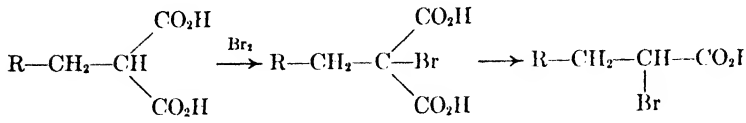
The yields in this reaction are usually good. Separation of the ammonium salt from the amino acid occasionally causes difficulty. In the earlier literature this was accomplished by treating the reaction product with silver or lead oxide to remove the halide followed with hydrogen sulfide to remove excess silver or lead. This method is unsatisfactory and should be avoided if possible. Ammonium bromide is moderately soluble in aqueous alcohol, ammonium chloride somewhat less so. It is often possible, therefore, to remove the ammonium halide by recrystallizing the amino acid from aqueous alcohol.[5, 6, 7] In other cases the insolubility of ammonium halides in glacial acetic acid has been utilized in purifying amino acids,[8] many of which are soluble in this acid.[9]

This method of preparing amino acids is useful, of course, only when the necessary α-halogen acids are readily available. Aliphatic α-bromo acids are prepared by direct bromination of the corresponding fatty acid.[5, 8]

$$R{-}CH_2{-}CO_2H + Br_2 \xrightarrow{PCl_3} R{-}\underset{\displaystyle Br}{\underset{|}{CH}}{-}CO_2H + HBr$$

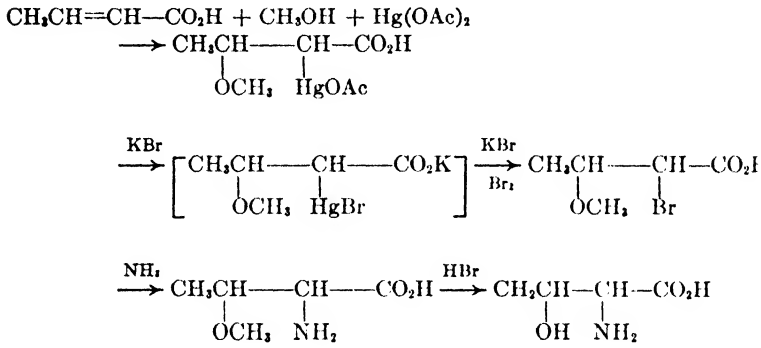
In other instances the corresponding malonic acid is employed.[6, 7] Because of the much greater ease of bromination of this type of compound it is possible to prepare bromo acids containing groups which would themselves be brominated under more drastic conditions.



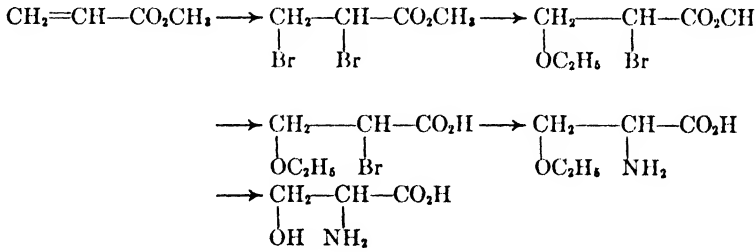


Glycine,[10] valine,[8] leucine,[5] isoleucine [6] and phenylalanine [7] are obtained in good yields by this method.

Special methods of preparation of α-bromo acids have been developed in the synthesis of more complex amino acids. Thus threonine is prepared as shown in the following equations: [11]

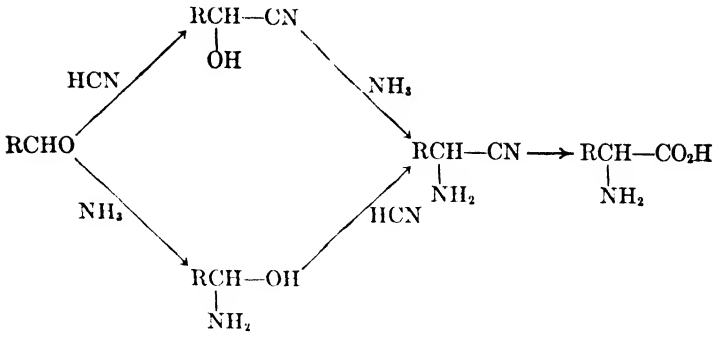


The two possible racemic forms (*dl*-threonine and *dl*-allothreonine) are obtained in these reactions and are separated through their N-benzoyl-O-methyl derivatives.[12] Serine may be obtained in a somewhat similar manner,[13] or more simply as shown below: [14]



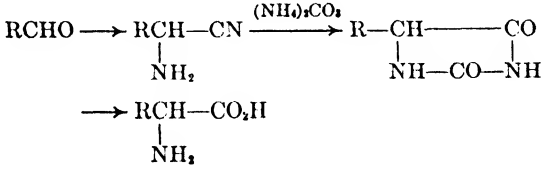
II. Strecker Synthesis

The Strecker synthesis provides a method of converting an aldehyde (or ketone) to an amino acid with one more carbon atom:

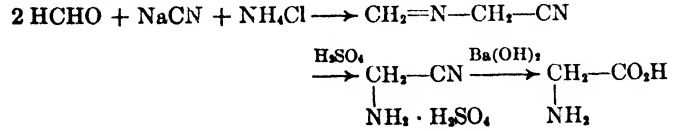


This reaction has been carried out in a variety of ways. The aldehyde in water, alcohol, or ether may be treated with ammonia or ammonium salts, followed by hydrogen cyanide or sodium cyanide. Or the cyanohydrin may be formed first and then treated with ammonia. In either case an aminonitrile is formed and is hydrolyzed directly to the amino acid by refluxing with strong acids or strong bases.

There are several disadvantages to this method. The necessary aldehydes are readily available in only a few instances. The yields are often poor, since the aldehyde tends to undergo side reactions. Hydrogen cyanide, of course, must be handled with extreme caution. In order to avoid its use mixtures of sodium cyanide and ammonium chloride have been employed. Such a procedure, however, introduces the problem of separating the amino acid from sodium chloride. Cocker and Lapworth [15] made several improvements in the Strecker synthesis. More recently Bucherer *et al.*[16] reported that an excellent yield of hydantoin is obtained if the reaction is carried out in the presence of ammonium carbonate. The hydantoin is readily isolated from the reaction mixture and may be hydrolyzed to the amino acid in a second step:



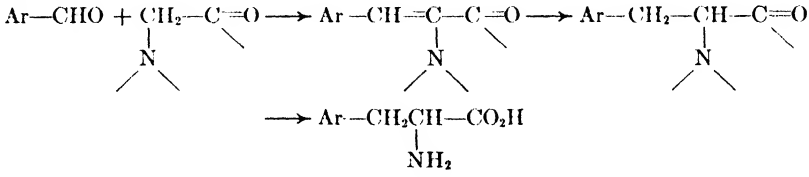
Despite these improvements and modifications, the Strecker method is rarely used except in the synthesis of glycine [17] and alanine.[18] In the former case an excess of formaldehyde is used and the aminonitrile is isolated as a crystalline formaldehyde condensation product,[19] thus facilitating isolation of the amino acid in the final step:



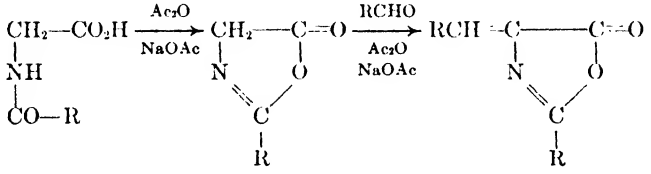
α-Aminoisobutyric acid [20] is prepared from acetone by the Strecker synthesis.

III. Condensation of Aldehydes with Glycine Derivatives

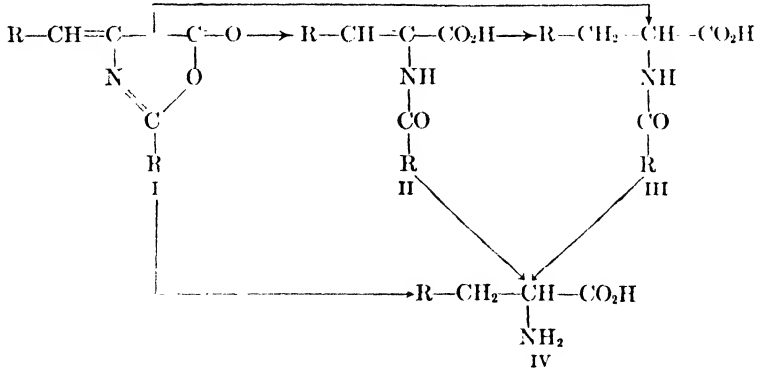
Aldehydes condense with a variety of glycine derivatives to give unsaturated compounds which are converted into amino acids by reduction and hydrolysis:



Perhaps the most useful of these reactions is the so-called "Erlenmeyer Azlactone Synthesis." In this reaction an acyl derivative of glycine (usually benzoyl or acetyl) is heated with the aldehyde, acetic anhydride and sodium acetate. The acylglycine is converted into an azlactone which has an extremely active methylene group and condenses rapidly with the aldehyde to give an unsaturated azlactone.



The azlactones are readily isolated from the reaction mixture and may be converted into the corresponding amino acids in several ways as shown in the equations:

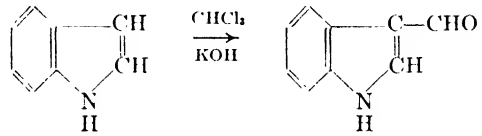


The azlactone (I) is hydrolyzed to the corresponding acylaminoacrylic acid (II) by alkali. Reduction of the double bond in either I or II may be effected with sodium amalgam,[21] red phosphorus and hydriodic acid in a

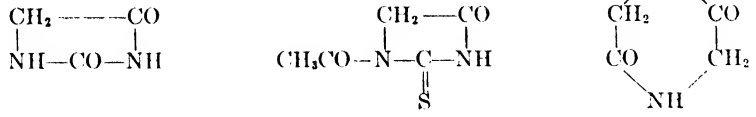
mixture of glacial acetic acid and acetic anhydride,[22, 23] or catalytically, using platinum as the catalyst.[24, 25] The second reagent, under the proper conditions, hydrolyzes the acylamino acid at the same time so that it is possible to go directly from an azlactone or acylaminoacrylic acid to the free amino acid.

The usefulness of this reaction is limited by the fact that aliphatic aldehydes do not give good yields in the condensation, since they tend to react with themselves rather than with the azlactone, and that ketones, with the exception of acetone, do not undergo the condensation. In the case of aromatic aldehydes the yields are generally excellent, and this method has given satisfactory results in the preparation of phenylalanine,[25, 26] tyrosine,[22, 23] thyroxine,[22] and a variety of amino acids related to thyroxine.

Tryptophane[27] and histidine[28] have been prepared by the azlactone synthesis. However, the method is only moderately satisfactory in these cases due to the difficulty of obtaining the necessary aldehydes. β-Indolealdehyde is prepared by treating indole with chloroform and potassium hydroxide:



As the yield in this reaction is only 30 per cent, the method of Snyder and Smith (described later) which avoids the use of β-indolealdehyde as an intermediate, is a much superior one for the synthesis of tryptophane. Imidazolealdehyde is obtained from citric acid by a six-step procedure which is quite tedious and laborious. Other glycine derivatives, such as hydantoin,[29] acetylthiohydantoin,[29] and diketopiperazine,[30] also condense readily with aromatic aldehydes to give unsaturated derivatives which may be reduced and hydrolyzed to the free amino acid.



Phenylalanine, tyrosine, tryptophane, and other aromatic amino acids have been prepared in this way. However, these compounds do not seem to offer any important advantages over the azlactone synthesis.

IV. Syntheses from α-Keto Acids and Their Derivatives

Several amino acids have been prepared from the corresponding α-keto acid or its oxime or phenylhydrazone. Most of these reactions have been developed recently. They are useful in special cases but are not applied generally in the synthesis of amino acids due in part to the fact that the nec-

essary α-keto acids (or derivatives) are not readily available except in a few instances.

A. From α-Keto Acids

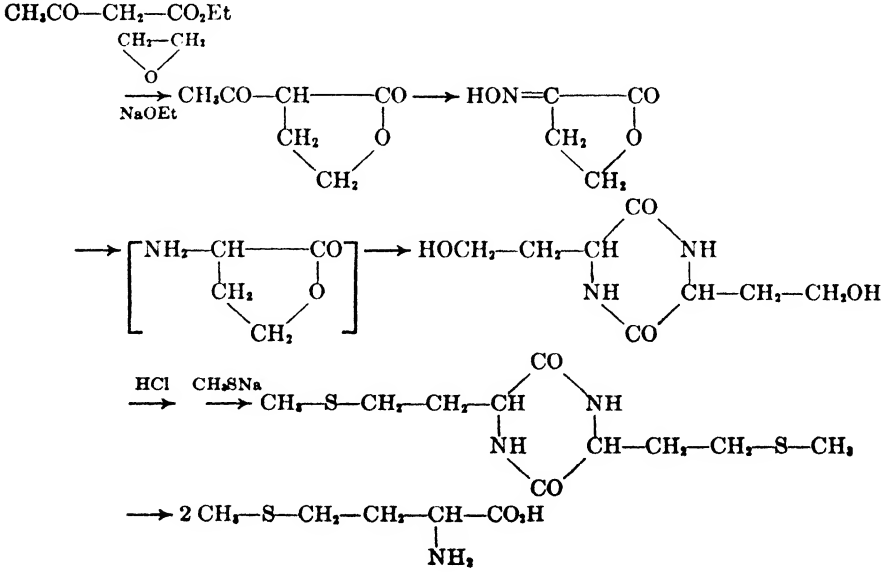
$$R{-}CO{-}CO_2H + NH_3 \xrightarrow[H_2]{Pd} R{-}\underset{\displaystyle NH_2}{\overset{|}{CH}}{-}CO_2H$$

This reaction, first studied by Knoop and Oesterlin,[31] was further developed by Schoenheimer and Ratner [32] as a means of introducing isotopic nitrogen (N^{15}) into the amino acid molecule without using a large excess of isotopic ammonia. The yields are usually good but the method is strictly limited by the lack of available α-keto acids. Alanine,[32] phenylalanine,[31] and glutamic acid [31] have been prepared in this way.

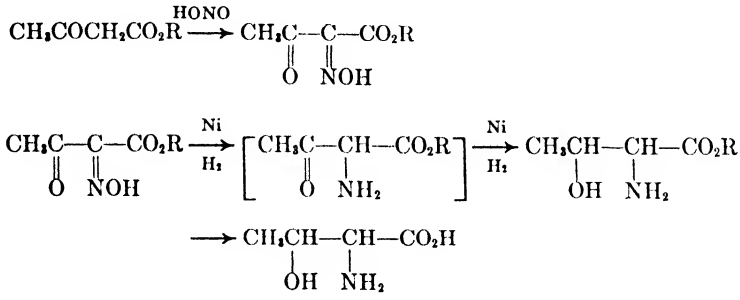
B. From Oximes of α-Keto Acids. Oximes of α-keto acids are reduced smoothly to the α-amino acid, using hydrogen and palladium-black or palladium-charcoal as a catalyst. The oximes may be prepared from the corresponding α-keto acids, but are more conveniently obtained directly from substituted acetoacetic esters.

$$CH_3CO{-}\underset{\displaystyle R}{\overset{|}{CH}}{-}CO_2R \xrightarrow[\text{or RONO}]{HONO} R{-}\underset{\displaystyle NOH}{\overset{\|}{C}}{-}CO_2R \xrightarrow[Pd]{H_2} R{-}\underset{\displaystyle NH_2}{\overset{|}{CH}}{-}CO_2R \longrightarrow R{-}\underset{\displaystyle NH_2}{\overset{|}{CH}}{-}CO_2H$$

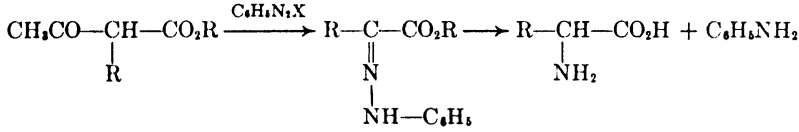
Acetoacetic esters react with nitrous acid (or nitrosylsulfuric acid) and with alkyl nitrites as shown in the equation. In the case of alkyl nitrites a catalyst such as sulfuric acid or sodium ethylate is used. The reaction proceeds smoothly and good yields are usually obtained. This reaction has been applied to a variety of compounds.[33] Of especial interest is the methionine synthesis developed by Snyder *et al.*[34]



Acetoacetic ester is not cleaved by nitrous acid but is converted into ethyl α-oximino-β-ketobutyrate. Adkins and Reeve[35] developed conditions for the catalytic reduction of this oxime to a mixture of threonine and allothreonine. Since the mixture is difficult to separate, this reaction is not particularly useful in the synthesis of pure threonine.



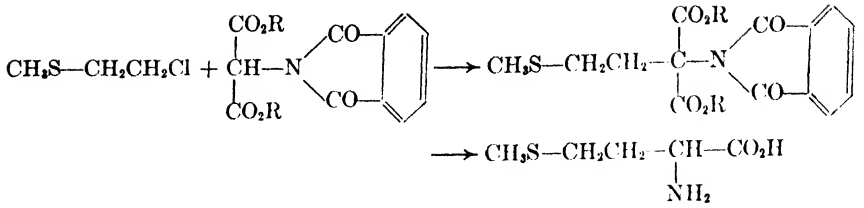
C. From Phenylhydrazones of α-Keto Acids. Substituted acetoacetic esters are converted into the phenylhydrazones of α-keto acids by the action of benzenediazonium chloride. Feofilaktov *et al.*[36] have utilized this reaction in the preparation of alanine, valine, leucine, and other amino acids.

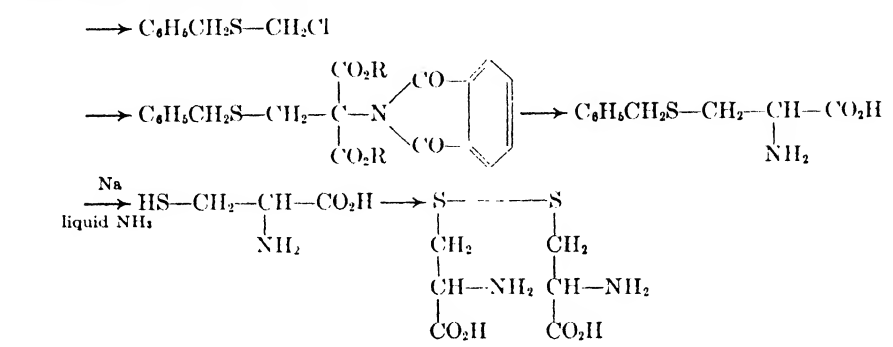


V. Syntheses from Derivatives of Aminomalonic Ester

The preparation of amino acids by the malonic ester synthesis has been described previously (See Method I). There are two disadvantages to this procedure. In the first place the alkylation of malonic ester with low molecular weight halides tends to produce disubstituted malonic esters. And, secondly, bromination of a malonic acid is not feasible if some other group in the molecule is attacked simultaneously. Both of these difficulties are avoided if a derivative of aminomalonic ester is employed. Historically, phthalimidomalonic ester is the first such compound to be studied. However, the use of phthalimidomalonic ester has certain drawbacks. The alkylation of phthalimidomalonic ester proceeds with difficulty and is usually effected in an alcohol-free solvent. Hydrolysis of the substituted ester is difficult. For these and other reasons this method has not found wide application.

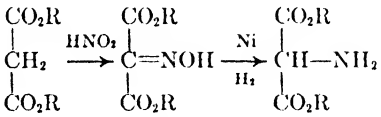
Phthalimidomalonic ester is prepared from bromomalonic ester and potassium phthalimide.[37] The following equations illustrate the use of the phthalimidomalonic ester method in the synthesis of methionine[38] and cystine.[39]



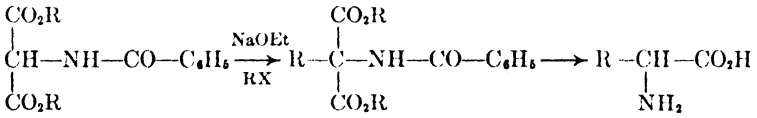


In the latter synthesis the cleavage of the benzyl group from the sulfur represents a general reaction which du Vigneaud and co-workers discovered and developed into an extremely useful tool in working with sulfur-containing amino acids.

With the advent of catalytic reduction methods, aminomalonic ester has become readily available.

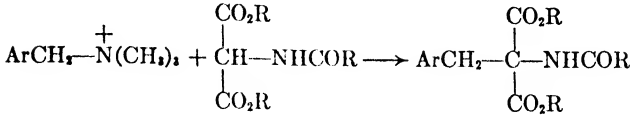


Aminomalonic ester itself is not suitable for use in the next step, since the free amino group would undergo alkylation. However, benzoylaminomalonic ester is easily obtained and has been used as an intermediate in the synthesis of several amino acids.[40]

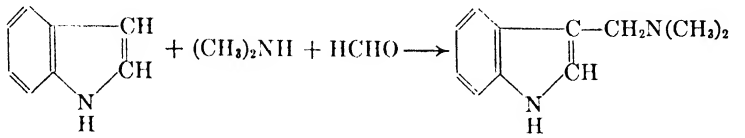


The alkylation proceeds smoothly, although the acylaminomalonic esters are not as easily alkylated as is malonic ester itself. The subsequent steps are effected in excellent yields.

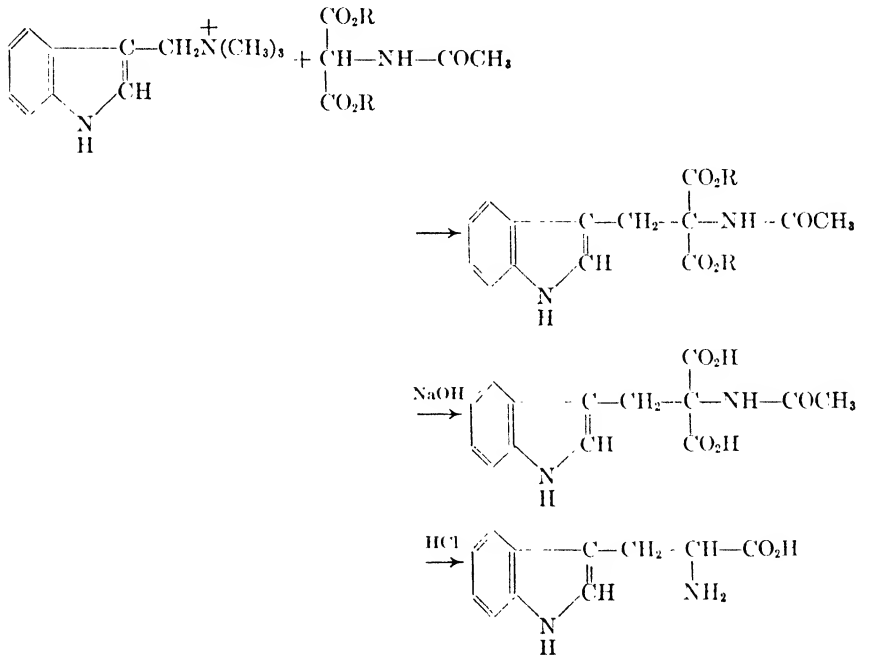
Snyder and Smith [41] have developed a unique and interesting variation of this method. They discovered that quaternary ammonium compounds of the type $Ar—CH_2—\overset{+}{N}(CH_3)_3$ will alkylate malonic esters as follows:



This discovery led to an excellent method for the preparation of tryptophane without the necessity of preparing β-indolealdehyde as an intermediate. Indole undergoes the Mannich reaction giving an excellent yield of gramine.



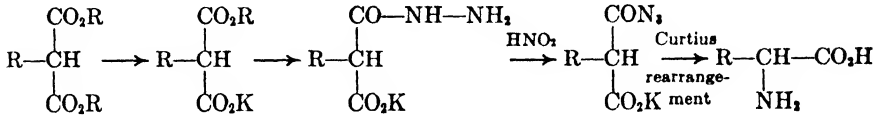
Methylation of the product gives the quaternary base (gramine methiodide) which can be used to alkylate acetylaminomalonic ester. The product yields tryptophane on hydrolysis.



Tryptophane has also been synthesized from indole through β-indolealdehyde as an intermediate (see Method III). However, the conversion of indole to β-indolealdehyde gives a poor yield. The method of Snyder and Smith is superior in that it does not involve β-indolealdehyde as an intermediate, and therefore gives a considerably better yield of tryptophane based on the original indole.

VI. Curtius Reaction

Substituted malonic esters may be converted into α-amino acids by the following reactions:

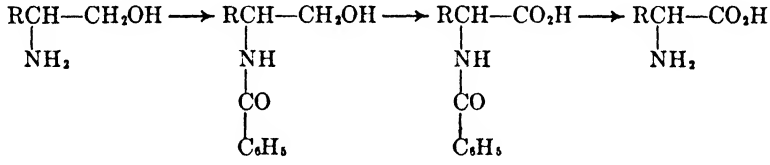


The conversion of a malonic ester to an amino acid is also effected by Method I which is more satisfactory in most instances. However, if the R group of the malonic ester is attacked by bromine, the Curtius rearrangement provides an alternative pathway which has found some use.[42]

VIa. Syntheses from Amino Alcohols.

Recently amino alcohols of the types shown below have become commercially available.

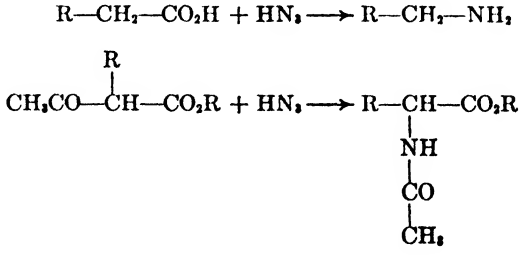
Billman and Parker (41 *a*) have developed a method for converting these compounds into α-amino acids as shown in the following equations:



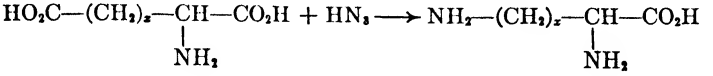
Each of the steps proceeds smoothly and the yields are good. Glycine, alanine, α-aminobutyric acid and α-aminoisobutyric acid have been prepared in this way.

VII. Schmidt Reaction

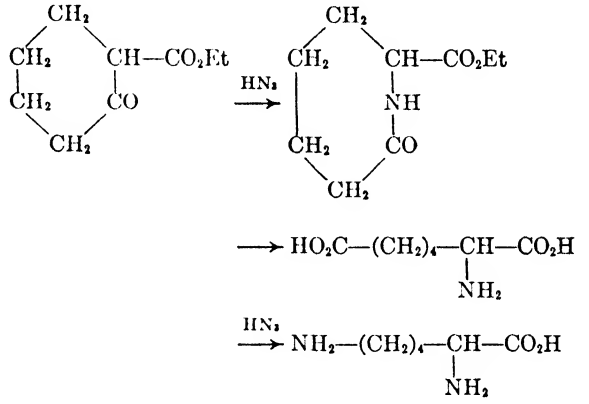
Hydrazoic acid reacts with carboxylic acids[43] and with acetoacetic esters,[43, 44] as shown in the following equations:



The fact that α-amino acids do not undergo this reaction makes possible an interesting synthesis of diamino acids.



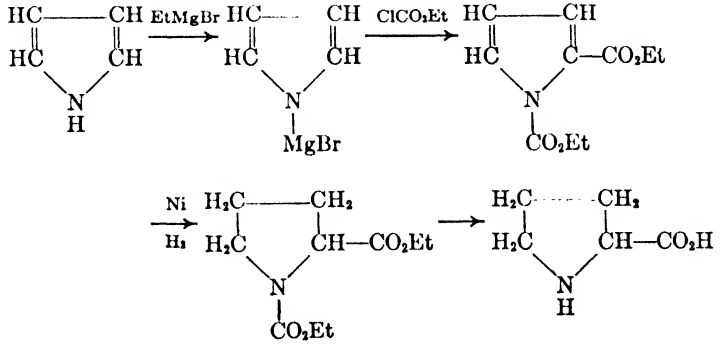
Thus lysine is prepared from α-aminopimelic acid which is in turn obtained from carbethoxycyclohexanone:



A serious drawback to this method is the fact that hydrazoic acid is extremely toxic and highly explosive if not properly handled.

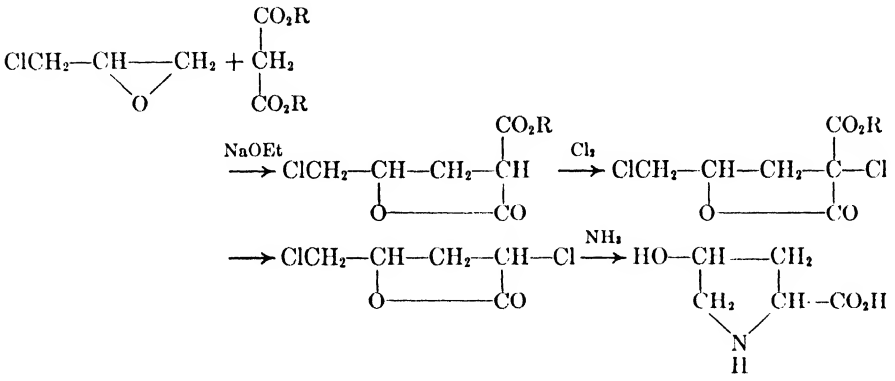
VIII. Special Methods

A. Proline. Signaigo and Adkins [45] developed an excellent method for preparing proline from pyrrole:



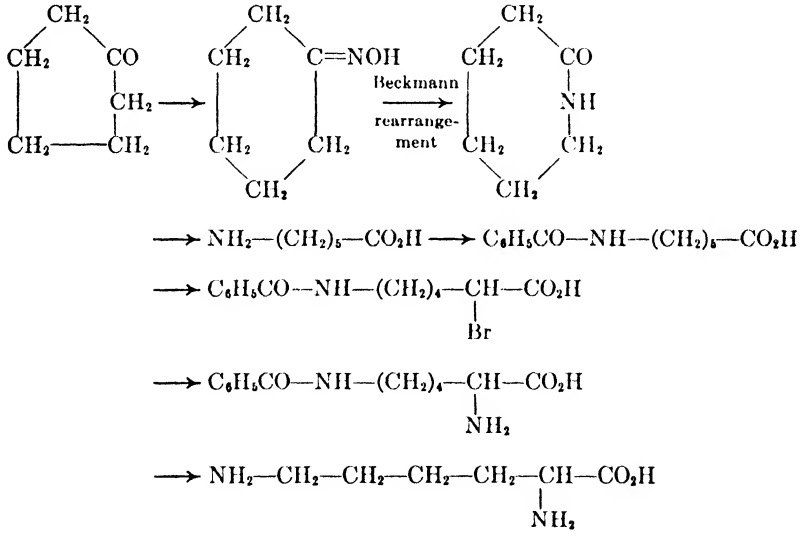
Reduction of pyrrole-α-carboxylic acid — the most obvious method — is not readily effected.

B. Hydroxyproline. Leuchs, *et al.*[46] developed the following method for preparing hydroxyproline:



A mixture of two racemic forms is obtained, one of which is identical with *dl*-hydroxyproline. More recently hydroxyproline has been prepared by method IVB.[47]

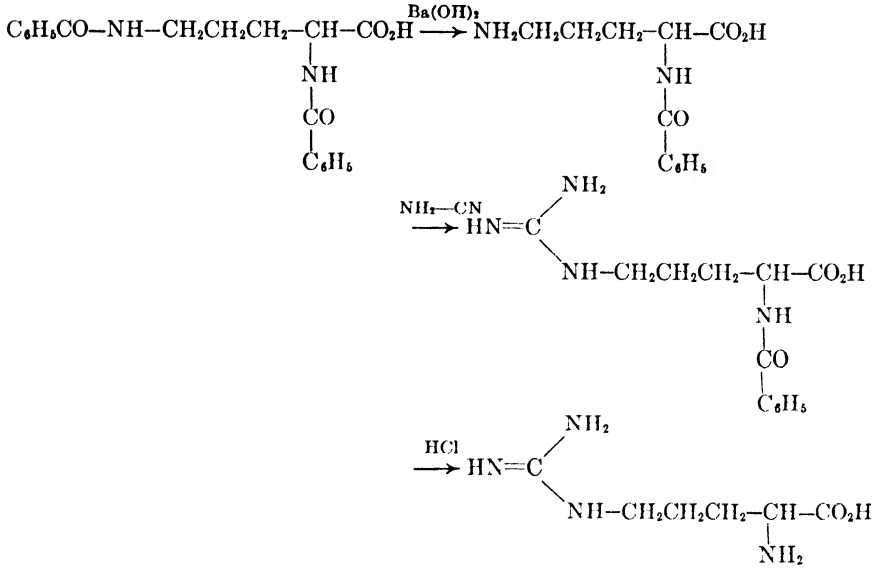
C. Lysine and Ornithine. Eck and Marvel[48] prepared lysine using cyclohexanone as the starting material:



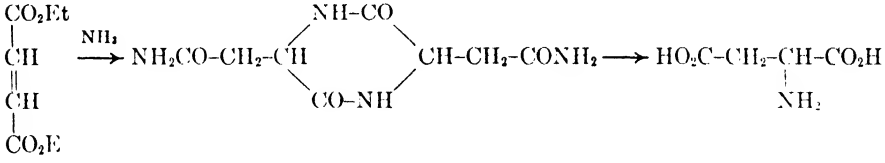
Although several steps are involved, each proceeds smoothly and the overall yield is satisfactory.

Ornithine has been prepared by applying the same series of reactions to cyclopentanone.[49]

D. Arginine. This amino acid is a derivative of ornithine and has been prepared from that compound by Sörensen, *et al.:* [50]

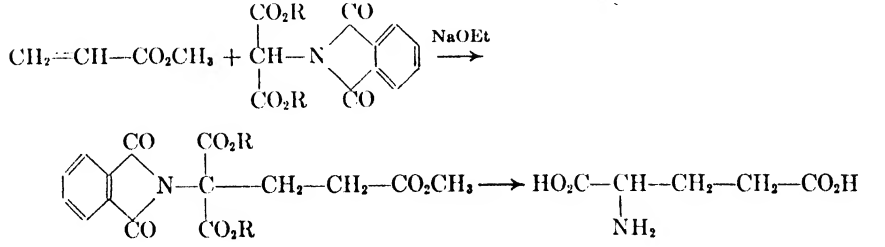


E. Aspartic Acid. Aspartic acid may be prepared in several ways (Methods IVB and V), but probably the most satisfactory is the reaction of ammonia with diethyl fumarate: [51]



The addition of ammonia to α, β-unsaturated acids is a general reaction which affords a useful method of preparing β-amino acids.

F. Glutamic Acid. The most satisfactory method of synthesizing glutamic acid is that of Marvel and Stoddard,[52] involving the addition of phalimidomalonic ester to methyl acrylate.



Summary

The best methods for the synthesis of the naturally occurring amino acids usually found in proteins are listed on the next page.

Glycine, I, II	Aspartic Acid, VIIIE
Alanine, I, II, IVA	Glutamic Acid, VIIIF
Serine, I, II	Lysine, VIIIC, VII
Threonine, I, IVB	Arginine, VIIID
Valine, I	Histidine, III
Norleucine, I	Proline, VIIIA
Leucine, I	Hydroxyproline, VIIIB
Isoleucine, I	Phenylalanine, I, III
Methionine, IVB, V	Tyrosine, III
Cystine, V	Tryptophane, III

Isolation of Certain Amino Acids

The isolation of amino acids from proteins involves two separate operations. First, the protein must be hydrolyzed to break the peptide bonds and free the amino acids, and secondly, the individual amino acids desired must be separated from this mixture. For a detailed description of hydrolysis of proteins see Chapter IV.

The naturally occurring amino acids may be divided into three groups: basic, neutral, and acidic. The basic amino acids are arginine, lysine, and histidine; the acidic are aspartic and glutamic acids. The remainder belong to the neutral or monoaminomonocarboxylic acid group.

The fractionation of protein hydrolyzates into these groups may be effected in a variety of ways. The basic amino acids are precipitated by the so-called "alkaloidal precipitants," such as phosphotungstic or phosphomolybdic acid. The dicarboxylic acids may be separated by virtue of the fact that their calcium salts are precipitated from an aqueous solution by the addition of alcohol, while the calcium salts of other amino acids are not. This is the basis of Foreman's method, which will be discussed later. The monoaminomonocarboxylic acids are extracted from an aqueous solution by butyl alcohol (Dakin's butyl alcohol method), but the basic and acidic amino acids are not. Finally, all three groups may be separated simultaneously by electrolysis of a protein hydrolyzate at the proper pH (electrical transport method).

Regardless of the method to be used it is customary to concentrate the neutralized protein hydrolyzate and filter off the insoluble amino acids. This fraction generally consists of tyrosine and leucine, and may also contain some cystine.

In separating amino acids from protein hydrolyzates, the unfractionated material may be used or one or more of the groups may be removed and worked up separately. Some of the better methods will be discussed and the isolation of certain amino acids will be considered. Excellent laboratory directions for the isolation of several amino acids are reported in *Organic Syntheses.*

Fischer's Ester Distillation Method. Today this is chiefly of historical interest as one of the earliest general methods for the separation of amino acids from protein hydrolyzates. Proposed by Fischer [53] and further investigated by Osborne and Jones [54] it depends on the fractional distillation *in vacuo* of the esters of the amino acids.

From the protein hydrolyzate after neutralization, tyrosine and leucine are partially removed by crystallization and glutamic acid is removed as the hydrochloride. Then the amino acids are esterified with absolute alcohol and gaseous hydrogen chloride, and glycine ester hydrochloride is removed. After removal of hydrogen chloride, the esters are fractionally distilled at low pressure. The residue contains the esters of the basic amino acids and some others. Sharp [55] has recently used a modification of this procedure in a study of the amino-acid composition of myosin.

Since the fractionation does not completely separate the esters, and losses are high, this method is little used today.

Dakin's Butyl Alcohol Method. This method, developed by Dakin,[56] is useful for separating the monoaminomonocarboxylic acids from the basic and acidic fractions. It depends on the solubility of the neutral amino acids in *n*-butyl alcohol saturated with water, in which the other two groups are practically insoluble.* A continuous extraction method is usually used and by modifying the conditions, such as temperature and pressure, the solubilities of some amino acids may be varied considerably. For example, hydroxyproline is extracted at atmospheric pressure but not at ten millimeters pressure. This method is still very valuable, both alone and in connection with other methods.

Electrical Transport Method. The electrical transport method for the separation of amino-acid mixtures into acidic, basic, and neutral fractions depends on the migration of these fractions under the influence of an electric potential. At the proper pH, the basic amino acids are positively charged and migrate through a membrane into the cathode compartment of a three compartment cell; the neutral acids remain in the center compartment, and the acidic amino acids, being negatively charged, migrate through a membrane into the anode section. First used by Ikeda and Suzuki,[57] this method was developed by Foster and Schmidt,[58] Cox, King, and Berg,[59] and Albanese [60] into a practical means of separating amino acids.

The electrolysis, if run at pH 5–6, causes lysine, histidine and arginine to enter the cathode compartment. By re-electrolysis of this fraction at pH 7–8 the more basic amino acids, arginine and lysine, may be almost completely separated from the less basic histidine and from contaminants from the first electrolysis. The acidic components (aspartic and glutamic acids) and proline and hydroxyproline can be recovered from the anode compartment.

* Sharp reported [55] that small quantities of basic and acidic amino acids are extracted by the butyl alcohol and recommends that these fractions be removed first.

The efficacy of this method is shown by the fact that Foster and Schmidt [58] obtained yields of arginine and lysine of 85 and 67 per cent respectively, using gelatin as the source. The electrical transport method has been widely used for group separations and for analytical purposes.

Kossel's Silver Salt Method. This method, depending on differences in solubility of the silver salts of the amino acids, was first used by Kossel.[61] Vickery and Leavenworth [62] employed it for the analytical determination of the basic amino acids, separating them first as silver salts and then isolating the individual acids with specific precipitants. This is probably used more as a quantitative method of determining the basic amino acids than as a preparative method.

Foreman's Dicarboxylic Acid Method. An effective method for the separation of the dicarboxylic amino acids from hydrolyzates is the precipitation of their barium or calcium salts with alcohol. By neutralizing the concentrated protein hydrolyzate with calcium or barium hydroxide and then pouring the solution into an excess of alcohol, barium or calcium glutamate and aspartate are precipitated, along with small amounts of other amino acid salts, which may be readily removed by a second or third reprecipitation of the salts from water.

This method was first used by Ritthausen in 1868 (see Chapter I). In 1914 Foreman [63] and subsequently Jones and Moeller [64] developed it as an analytical procedure. Glutamic acid is separated as its hydrochloride and aspartic acid as its copper salt. It is now widely applied to remove these acids from the hydrolyzates of proteins to obtain the neutral or basic amino acids.

Shryver's Carbamate Method. Shryver [65, 66] has described a method for partial separation of hydrolyzates which depends on the formation of carbamino derivatives insoluble in alcohol when a solution of amino acids is treated with carbon dioxide in the presence of barium hydroxide. Methods are described for the isolation of glycine, proline, hydroxyproline and the dicarboxylic amino acids.

Selective Adsorption Methods. The search for materials which will exert a selective adsorption effect on amino acids has been carried on for some time. This method is not at present developed to the extent of offering a practical means of isolating amino acids, but it shows some promise.

Felix and Lang,[67] Sadikov,[68] and Whitehorn [69] reported that Permutit selectively adsorbs the basic amino acids. Mashino [70] obtained similar results using Japanese acid clay. Calcium hydroxide was used as the eluting agent. Tiselius [71] and Wachtel and Cassidy [72] used activated carbon as the adsorbent. Wachtel and Cassidy have separated quantitatively mixtures of *l*-tyrosine and *dl*-leucine, and of *dl*-phenylalanine and *dl*-leucine.

Turba,[73] using activated Fuller's earth, separated the basic amino acids from the monoamino acids and from one another.

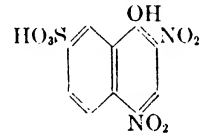
Ion-exchange Resins. The commercial availability of synthetic ion-exchange resins such as the "Amberlites" has stimulated interest in their use for amino-acid separations. Block [74] reported that these resins have proved useful for large-scale separation of the basic amino acids from protein hydrolyzates.

Insoluble Amino-acid Salts. The ideal method of amino-acid isolation would, of course, consist of using a specific precipitant for each amino acid. It is well known that several of the amino acids form insoluble salts with various types of reagents and many of these salts have proved useful for isolation purposes.

Complex Inorganic Salts. Kapfhammer and Eck,[75] using Reinecke salt $[(NH_3)_2Cr(CNS)_4] \cdot NH_4$, have described the isolation of proline and hydroxyproline. Specific inorganic reagents for several amino acids have been developed by Bergmann and co-workers.[76, 77] Glycine gives well crystallized double salts with potassium trioxalatochromiate, $Cr(C_2O_4)_3K_3 \cdot 3H_2O$, which have been used for its estimation. Dioxalatodipyridinochromiatic acid, $[Cr(C_2O_4)_2 \cdot (C_5H_5N)_2] \cdot H$, has been used for alanine determinations.

Many heavy-metal salts form insoluble complexes with amino acids, and several of these have been used for separation and analysis of hydrolyzates. Mercury, silver, and copper salts are used extensively, as are phosphotungstic and phosphomolybdic acids.

Among the most useful reagents for isolation of amino acids as insoluble salts are the aromatic sulfonic acids. One of the first of these to be used was flavianic acid or 1-naphthol-2,4-dinitro-7-sulfonic acid:



Kossel and Gross [78] first used this as a precipitant for arginine. Using a modified procedure, Pratt [79] reports yields up to 90 per cent in the isolation of arginine from gelatin. Vickery [94] made an exhaustive study of the conditions under which flavianic acid may best be used as a reagent for the isolation and quantitative determination of arginine.

Extensive investigation of other aromatic sulfonic acids has yielded many useful methods for amino-acid isolation. Bergmann and co-workers [80, 81, 82] reported studies with several sulfonic acids and developed isolation methods for leucine and phenylalanine from hemoglobin and serine and alanine from silk fibroin. Vickery [83] used 3,4-dichlorobenzenesulfonic acid to isolate histidine from hemoglobin hydrolyzates.

A valuable feature of the sulfonic acid salts is the fact that they usually show sharp melting points, which is valuable for identification and as an index of purity.

Among other reagents forming insoluble amino acid salts is picric acid which has been used [84] in the isolation of lysine.

Isolation of Individual Amino Acids

Cystine. Cystine is most conveniently isolated from hair or wool [85, 86]. Following acid hydrolysis and removal of excess acid, the hydrolyzate is brought to the isoelectric region of cystine (pH 3–6) with sodium acetate and the crude precipitate of cystine is removed. To purify the crude material it is dissolved in dilute acid and reprecipitated with sodium acetate.

Tryptophane. Tryptophane may be isolated from casein, using enzymic hydrolysis.[87] A crude tyrosine fraction is filtered off and the tryptophane precipitated with mercuric sulfate in sulfuric acid. The precipitate is decomposed with hydrogen sulfide and the tryptophane removed by extraction with butyl alcohol, from which it is recovered.

Tyrosine. Tyrosine may be prepared from casein along with tryptophane.[87] The tyrosine is precipitated by adjusting the reaction to the isoelectric region (pH 5–7). The crude tyrosine is freed of leucine by extraction with hot glacial acetic acid in which leucine is soluble.[88]

Histidine. Histidine has been prepared by precipitation with mercuric chloride [89, 90] and by precipitation with mercuric sulfate [91] after separating the basic amino acids by electrical transport. Probably the best method at present is that of Vickery,[83] who used 3,4-dichlorobenzenesulfonic acid, precipitating the insoluble sulfonate directly from the hydrolyzate.

Arginine. Arginine is often isolated by use of the dinitronaphtholsulfonate.[78, 79] Usually this is used in conjunction with other methods which separate the basic amino acids from the hydrolyzate. Another good method is that of Bergmann and Zervas,[91] as modified by Brand and Sandberg,[92] in which the arginine is precipitated from alkaline solution as the benzylidine derivative.

Serine and Alanine. Serine is easily isolated from silk fibroin by the method of Bergmann.[52] Glycine is removed from the hydrolyzate as the 5-nitronaphthalene-1-sulfonate and alanine as the azobenzene-*p*-sulfonate. The serine is then precipitated with *p*-hydroxyazobenzene-*p'*-sulfonic acid and recovered by decomposition of the insoluble salt with barium acetate.

Alanine may also be isolated in this procedure by recovery from its insoluble sulfonate.

Lysine. Lysine was, until recently, best isolated by the picrate method of Rice,[84] which avoided the tedious electrical transport method by using direct precipitation. However, the method fails to work with gelatin, according to Kurtz,[93] who has proposed an alternative method. Following hydrolysis and neutralization, arginine is precipitated as the dinitronaphtholsulfonate, and the copper salts of the remaining amino acids are formed. Benzoylation then precipitates the copper salt of ϵ-benzoyllysine which is converted to ϵ-benzoyllysine and then to lysine dihydrochloride.

The ion exchange method of Block [74] may serve as the basis for a simple method of preparing lysine because of the ease with which the basic amino acids are separated from the other fractions.

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Chapter VI

Methods of Analysis for Amino Acids and Proteins

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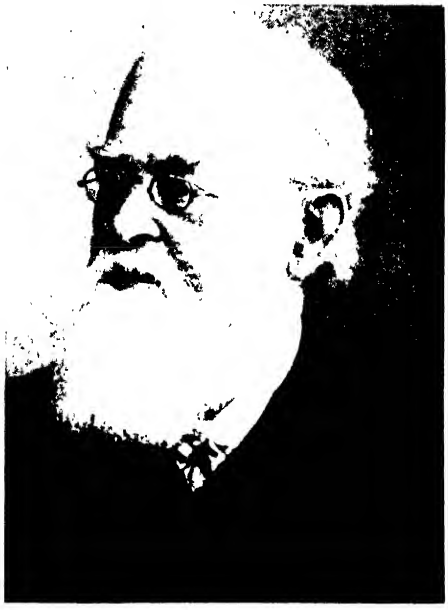


Heinrich Ritthausen

Born in Armenruh near Goldberg in Silesia in 1826 and died in 1912. He worked on vegetable proteins and carbohydrates and discovered glutamic acid in proteins and raffinose in cottonseed.

Introduction

Analytical methods for the determination of amino acids are important for evaluating the amino-acid content of proteins. To determine the value of a protein as a nutriment, or as a source of amino acids for commercial, clinical, or scientific purposes, it is necessary to be able to determine its amino-acid content. It is also hoped that by accurate methods of analysis, it may be possible to find the source of the individual properties of such important proteins as enzymes and hormones.

Methods for the analysis of amino acids in blood and urine have a clinical value which may be enhanced with increasing knowledge of the physiological functions of the amino acids. These methods have a field of usefulness in the diagnosis of certain diseases, particularly of the liver.

Analysis of the blood amino-acid level is important for the proper control of amino-acid therapy.

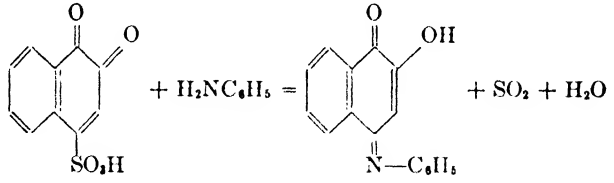
Two types of analytical methods are desirable. One type consists of estimating the general level of all the amino acids, irrespective of individuality. Such methods depend upon the application of some chemical reaction which is common to all the constituents of proteins. A second type consists of methods that are specific for a single amino acid and is of paramount interest in the evaluation of the amino-acid make-up of proteins and protein derivatives.

Methods for Determining Total Amino-Acid Content and Their Application to Blood and Urine

The Folin Colorimetric Estimation of Amino Nitrogen with β-Naphthaquinone Sulfonate and its Modifications

The reaction between amino acids and 1,2-naphthaquinone-4-sulfonate was discovered by Folin [1] and was made the basis of a method for estimating blood and urine amino-acid nitrogen. The method has since undergone considerable modification and improvement at the hands of a number of investigators. Danielson [2] improved the accuracy of the method, and Sahyun [3] speeded up the reaction by heating the reaction mixture during color development.

The reaction between the amino acids and the quinone is believed to be similar to the reaction between this compound and aniline. The equation for this is



An extensive survey of compounds other than amino acids which react with the quinone and yield similar colors has recently been carried out by Frame, Russell, and Wilhelmi.[4] The interfering effect of ammonia was observed by Folin. The more recent work shows that similar but less intense colors than those given by the amino acids are yielded by ammonia, primary aliphatic amines, and some secondary amines. Primary aromatic amines yield as much color as the amino acids. These include the various sulfanilamides. In addition, uric acid and allantoin react to give red-colored substances in acid solution. A comparison of these two substances with amino-acid standards showed that 28 per cent of the uric acid nitrogen and 13 per cent of the allantoin nitrogen reacted as amino nitrogen.

The interfering effect of ammonia, uric acid, and allantoin, is of no great significance in the case of blood and most tissues. They probably render

the method of little quantitative value for the estimation of amino-acid nitrogen in urine.

Outline of Procedure for Blood. The modification of the method developed by Sahyun seems to be the most satisfactory for general usage. The details of the procedure are as follows:

Reagents: 0.1*N* NaOH

2 per cent solution of *sodium borate.*

0.25 per cent solution of *phenolphthalein.*

0.5 per cent solution of sodium β-naphthaquinone sulfonate, prepared freshly before using.

Do not use a solution that has stood for more than 15–20 minutes.

Special Acetic Acid-Acetate Reagent: Dilute 100 ml of 50 per cent acetic acid with an equal volume of 5 per cent sodium acetate solution.

Sodium Thiosulfate: A 4 per cent solution of $Na_2S_2O_3 \cdot 5\,H_2O$.

Stock Standard Solutions: Samples of glycine and glutamic acid are dried to constant weight over sulfuric acid in a vacuum desiccator. Accurately weigh 267.5 mg of glycine and dissolve in 500 ml of 0.07*N* HCl containing 0.2 per cent of sodium benzoate as preservative. A stock solution of glutamic acid is prepared in the same way, using 525 mg of glutamic acid to 500 ml of 0.07*N* HCl containing 0.2 per cent of sodium benzoate. The stock solutions thus prepared will each contain 0.1 mg of amino acid nitrogen per ml, and will keep indefinitely.

Mixed Standard of 0.1 Mg Amino Acid Nitrogen per ml: Introduce 5 ml of the stock glycine standard and 5 ml of the stock glutamic acid standard into a 100-ml volumetric flask. Make up to volume with distilled water; mix thoroughly, add a few drops of chloroform and keep in a refrigerator when not in use. It is preferable to prepare the diluted standard once a week.

Procedure for Blood: Blood filtrates obtained by the Folin and Wu tungstate method or by Somogyi's zinc hydroxide precipitation method are excellent for determining total amino-acid nitrogen. Filtrates obtained by Benedict's method are also satisfactory.

To estimate the amino-acid nitrogen, introduce 3 ml of the blood filtrate, or a volume containing about 0.03 mg amino nitrogen into a 25–30-ml test tube. Simultaneously, measure 3 ml of the standard amino-acid solution (0.03 mg amino nitrogen) into another test tube. Add one drop of phenolphthalein to each tube and, while shaking, add 0.1*N* NaOH, 1 drop at a time until 1 drop brings about a permanent pink color. Add 1 ml of sodium borate solution, followed by 2 ml of freshly prepared sodium β-naphthaquinone sulfonate reagent; mix by gently rotating the tubes and adjust the samples to an equal volume by the addition of distilled water. It is desirable to keep the volume as small as possible. Immerse the tubes in a boiling water bath for 3 minutes, remove, and cool to room temperature in a stream of running water. To each tube, first add 2 ml of

acetic acid reagent, mix, and then add 2 ml of 4 per cent sodium thiosulfate solution. Dilute the contents of each tube to the 25-ml mark, mix, and compare the colors in a suitable colorimeter.

When the color comparison is carried out in a Duboscq type (or Klett) colorimeter, the standard for comparison that is selected should be the one that most closely approaches the unknown in its concentration. Sahyun considers that readings of the unknown between 15 and 28, when the standard is set at 20, give reliable results. If the reading is stronger than 15, or weaker than 28, either the unknown is read against the next standard that gives a closer match, or the determination is repeated with another more suitable filtrate volume.

Micro Method: A colorimetric method which may be used to determine 4 to 40 γ of amino nitrogen per sample has been developed by Frame, Russell, and Wilhelmi.[4] The authors state that duplicate analyses may be made upon 0.2 ml of blood. This method was made possible by using a photoelectric colorimeter to determine the intensity of color.

Determination of Amino-Acid Nitrogen in Urine. No high degree of accuracy can be attained with the colorimetric method for determining the amino-acid nitrogen in urine. As has already been mentioned, ammonia, uric acid, and allantoin give interfering colors. The ammonia can be removed by adsorption on Permutit, but this procedure also causes a loss of considerable amino-acid nitrogen.

In the procedure described by Sahyun, two dilutions of urine are prepared in the ratios of 1 : 10 and 1 : 20 in order to take care of the fluctuations of urinary amino-acid nitrogen. To carry out the analysis, introduce 5 ml of urine each into a 50-ml and a 100-ml volumetric flask and dilute to volume. Mix well and transfer to Erlenmeyer flasks. Add 2-3 gm of Permutit and shake gently for 5 minutes. Decant into clean flasks and again add the same amount of Permutit, shake, and let stand for 10 minutes before filtering. It is advisable at this point to test 1 ml of the filtrate with Nessler's solution to ascertain the complete absence of ammonia.

From the clear, ammonia-free diluted urine, transfer 2-ml and 3-ml samples into graduated 25- or 30-ml test tubes. Simultaneously prepare two standards from the 0.01 mg per ml amino acids standard given above, one containing 0.03 mg and another 0.05 mg of amino-acid nitrogen. Carry out the rest of the determination in the same manner as described in the Sahyun method for blood.

The Van Slyke Nitrous Acid Reaction

Primary aliphatic amino groups react with nitrous acid to yield N_2, according to the following reaction:

$$RNH_2 + HNO_2 = ROH + H_2O + N_2$$

The reaction can be used to measure the amino-acid nitrogen of blood and of urine. The method is not highly specific, since amines other than

the amino acids and certain other compounds, such as ammonia and urea, also react with nitrous acid to yield N_2. However, the NH_2 groups of α-amino acids react quantitatively in 3 or 4 minutes at room temperature; while the other substances react much more slowly. About 25 per cent of the ammonia and 6 to 7 per cent of urea react with nitrous acid in the time required for complete reaction of α-amino acids.

An estimation with nitrous acid on blood or urine, under appropriate conditions, gives mainly the amino-acid nitrogen; but also includes some N_2 derived from amines and possibly other unknown compounds.

Van Slyke [5] has adapted the nitrous acid reaction to the manometric gas apparatus, thus greatly increasing its sensitivity and making it possible to perform a gasometric amino nitrogen determination directly on 5 ml of a Folin-Wu tungstic acid blood filtrate. Formerly, it was necessary to concentrate the filtrate to a smaller volume.

The reaction is carried out by mixing sodium nitrite, acetic acid, and the amino-acid containing solution. In the manometric apparatus, acetic acid and the amino-acid solutions are mixed together in the reaction chamber, freed of air, and the $NaNO_2$ is then added in saturated solution. The saturated nitrite solution need not be free of dissolved air before it is used. Because of its high salt content, the amount of air dissolved by this solution is negligible.

The total analysis requires about 12 to 15 minutes. The maximum amount of amino nitrogen that can be determined in a sample is about 0.6 mg which, at a 2-ml volume, yields nitrogen gas giving a pressure of about 400 mm Hg. The minimum measurable quantity is about 0.4 γ, which yields nitrogen gas giving 1 mm pressure at 0.5 ml volume. It is not feasible to describe the method in detail because of the space required. The reader is referred to the original publications for the details of the method.

Determination of Amino-Acid Nitrogen in Blood. In blood filtrates, not only the amino acids, but also the urea reacts measurably with nitrous acid. In the time required for complete reaction of the α-amino acids, about 7 per cent of the urea nitrogen is decomposed. In human blood without pathological urea retention, the amino nitrogen can be determined without preliminary removal of the urea, a correction of 0.07 of the urea nitrogen being subtracted from the total nitrogen obtained by the nitrous acid reaction. When there is gross urea retention (blood urea nitrogen about 50 mg per 100 ml) it is desirable to remove the urea with urease and boil off the resulting ammonia before determining the amino-acid nitrogen.

In the case of blood of normal urea content, the analysis is carried out on 5 ml of blood filtrate prepared by the tungstic acid method of Folin and Wu and representing 0.5 ml of blood. This is pipetted into the chamber of the gas apparatus and analyzed as described above. The time of reac-

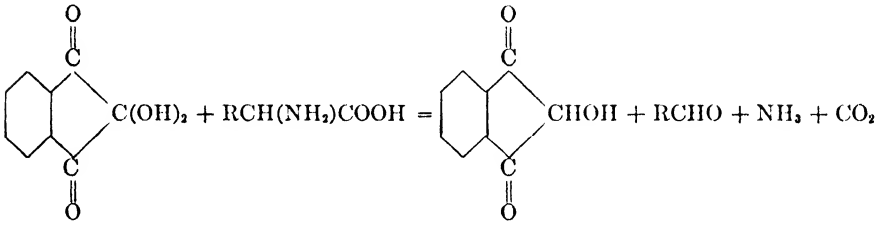
tion, measured from the moment the sodium nitrite solution is run into the chamber to the end of the period of shaking, varies with the temperature and must be regulated carefully, in order that the proportion of urea decomposed shall approximate the constant value of 0.07 allowed for. At 20° the required shaking time is 4 minutes, at 25° it is 3 minutes.

Total (free and conjugated) Amino-Acid Nitrogen in Urine.[6] The amino nitrogen in urine represents both that of free amino acids and of conjugated amino acids, as in hippuric acid.

For an accurate estimation of the amino nitrogen of urine, it is necessary to remove both urea and ammonia. Urea is first hydrolyzed to ammonia by heating a sample of urine with sulfuric acid. The sulfuric acid is next precipitated by the addition of calcium hydroxide and the resulting calcium sulfate is removed by filtration. The filtrate is rendered alkaline and ammonia is driven off by evaporation.

The Ninhydrin-Carbon Dioxide Reaction

When α-amino acids are boiled in water with an excess of ninhydrin (tri-ketohydrindene hydrate) they quantitatively evolve the CO_2 of their carboxyl groups in a few minutes. The reaction that takes place may be represented by the equation shown below:



Van Slyke and co-workers [7-11] have developed highly accurate methods for determining amino acids by estimation of the CO_2 evolved in this reaction.*

This is the most specific of the known methods for the analysis of total amino-acid content. Van Slyke and co-workers [9] state: "The reaction is specific for free amino acids in that it requires the presence, in the free unconjugated state, of both the carboxyl and the neighboring NH_2 or NH—CH_2 group." The reaction goes to completion with amino acids having a primary α-NH_2 group, with proline, hydroxyproline, and with sarcosine (mono methyl glycine). Some degree of reactivity of the —COOH group is retained if the —NH_2 is in the β position. Thus aspartic acid evolves all the CO_2 of both of its —COOH groups. On the other hand, the distal —COOH group of glutamic acid does not react.

* MacFayden [10b] has developed a manometric method for determining the ammonia evolved from primary α-amino groups of amino acids when they react with ninhydrin in boiling aqueous solution at pH 2.5.

The ninhydrin-CO_2 reaction serves to differentiate free amino acids from peptides more sharply than is possible by any other known method.

It might be expected that organic carboxylic acids would interfere with the method. This is not the case for unsubstituted organic acids or for hydroxy acids. Such acids as acetic, lactic, and citric do not lead to evolution of CO_2. Keto acids such as pyruvic and acetoacetic do react, but they decompose with loss of CO_2 when boiled with water; therefore they can be completely decomposed by preliminary boiling so as not to interfere with measurement of CO_2 from the amino acids. Ascorbic acid also reacts to a slight degree, but the amounts of ascorbic acid present in blood and urine are too slight to have a significant effect on amino acid determination unless large doses have been ingested. The reader is referred to the original publications for the details of the manometric methods.[8-11] The titration procedure is described below:

Titration Method for the Determination of Free Amino Acids by the Ninhydrin-Carbon Dioxide Reaction. A relatively simple way of determining amino acids by the ninhydrin-carbon dioxide reaction is to transfer the evolved CO_2 to standard barium hydroxide and titrate the excess. This requires no costly or elaborate equipment. Descriptions of methods to carry out the analysis in this manner have been published by Christensen, West, and Dimick [12] and by Van Slyke, MacFadyen, and Hamilton.[9a] According to the method of Van Slyke *et al.*, the transfer of the CO_2 to the barium hydroxide is carried out by distillation *in vacuo*. This requires 2 to 3 minutes. Barium chloride is added to the barium hydroxide to insure complete precipitation of the barium carbonate. The apparatus consists of a pair of small Erlenmeyer flasks attached to a U-tube. The reaction, distillation, and titration are all carried out in the apparatus shown in Fig. 1.

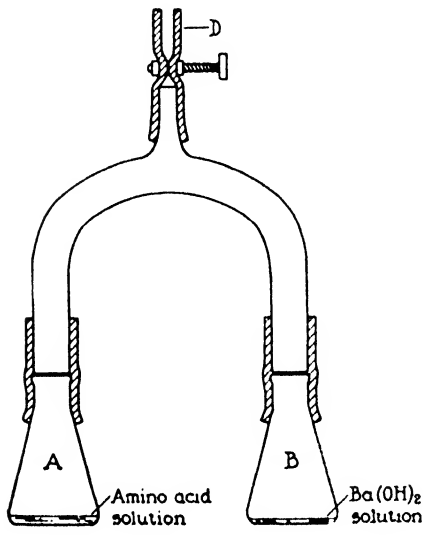


FIG. 1. Apparatus for evolution and distillation of carboxyl CO_2. From Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).

The titration method yields results of the same order of constancy as the manometric method for macro- and microanalysis. For submicroanalysis (carboxyl carbon under 0.1 mg), the titration is less accurate than the manometric method. The manometric procedure requires fewer precautions against atmospheric CO_2, is more accurate for minimal amounts of amino acids, and is free from the necessity of continual restandardization of solutions.

Apparatus. The apparatus consists of two Pyrex 25-ml Erlenmeyer flasks and a U-tube (Fig. 1). The U-tube and necks of the flasks should have an external diameter of 16–17 mm. The connection is made by rubber tubing of $\frac{1}{2}$ inch (12 mm) bore and $\frac{3}{16}$ inch wall thickness. For precautions with regard to selection and cleaning of the rubber tubing see page 633 of reference 8. If the 25-ml flasks have flanges, these should be cut off or pushed in after softening the glass in a burner. The 25-ml flasks may be replaced by the special test-tubes used for manometric analysis (Fig. 1, A, reference 8).

Obtain a 5-ml burette accurate to 0.01 ml. For submicroanalysis a Rehberg micro-burette of 200 cubic mm capacity is needed.

A reservoir of CO_2-free air is required. One is conveniently made of two aspirator bottles of 2 liters each, with their lower openings connected by a rubber tube. The bottles are charged with 2.5 liters of 10 per cent NaOH solution. The upper opening of one bottle is closed by a soda-lime tube, that of the other by a perforated stopper connected with light rubber tubing ending in a glass capillary from which CO_2-free air can be drawn. When all the air from this bottle has been used, the soda-lime tube and air exit tube on the two bottles are interchanged, the bottle full of solution is elevated, and CO_2-free air is drawn from the other, after it has been shaken to assure absorption of all the CO_2. The bottles are marked at 200-ml intervals for convenience in measuring air outflow.

Calibrated *glass spoons* are to be used for the measurement of 50- and 100-mg charges of ninhydrin and citrate buffer.[8, 13]

A water bath is also required.

Reagents. *Ninhydrin pulverized.* This may be purchased from the Eastman Kodak Co. under the name Triketohydrindenehydrate.

Citrate Buffers. The buffer for pH 4.7 consists of 17.65 gm of $Na_3C_6H_5O_7 \cdot 2\,H_2O$ and 8.40 gm of $C_6H_8O_7 \cdot H_2O$. The buffer for pH 2.5 consists of 2.06 gm of trisodium citrate and 19.15 gm of citric acid. The constituents are first finely pulverized separately, then mixed in the proper proportions and ground together.

Approximately 0.25N $Ba(OH)_2$ solution containing 2 per cent of $BaCl_2$. For macroanalysis. A saturated solution of $Ba(OH)_2$ is titrated and diluted to a concentration of 0.3*N*. Five volumes of this solution are mixed with 1 volume of a neutral solution of $BaCl_2$ containing 12 gm of $BaCl_2 \cdot 2\,H_2O$ per 100 ml.

Approximately 0.125N $Ba(OH)_2$ solution containing 2 per cent of $BaCl_2$. For microanalysis. A 0.15*N* $Ba(OH)_2$ solution is prepared, and 5 volumes are mixed with 1 volume of the 12 per cent $BaCl_2$.

Approximately 0.0155N $Ba(OH)_2$ solution containing 10.5 per cent of $BaCl_2$. For submicro analysis. 1 volume of the 0.125*N* $Ba(OH)_2$ is diluted with 7 volumes of the 12 per cent neutral $BaCl_2$ solution.

Standard 0.1428, 0.07138, and 0.02855N HCl (N/7, N/14 and N/35 × 14.00/14.01). These concentrations are chosen because 1-ml portions are equivalent to 1, 0.5, and 0.2 mg of carboxyl nitrogen respectively, which is more frequently calculated than carboxyl carbon.

Approximately 10 per cent NaOH for use in the reservoir of CO_2-free air.

Caprylic alcohol, as anti-foam.

Indicators. 1 per cent phenolphthalein in 95 per cent alcohol; 0.04 per cent cresol red solution in water.

Sodium veronal buffer of pH 8.0. This buffer is prepared from a stock solution containing 10.3 gm of sodium veronal in 500 ml of water. Seven ml of stock solution is mixed with 4 ml of *N*/14 HCl. The solution is used as a color standard for the end-point in the submicrotitration.

Procedure. *Reaction with Ninhydrin.* The amino acid solution is placed in flask A (Fig. 1). If the analysis is submicro, the solution should not exceed 2 ml in volume; for micro- or macroanalyses the volume may be as high as 5 ml. Fifty mg of the appropriate citrate buffer (usually pH 2.5) is added if the volume of the sample is 2 ml, and 100 mg if the sample volume is between 3–5 ml. A few pieces of Alundum are added to prevent bumping, and a drop of caprylic alcohol to prevent foaming. The reaction vessel is then boiled for 20 to 30 seconds to drive off preformed CO_2. If the presence of α-keto acids is suspected, heating is continued in the water bath as long as may be necessary to complete the evolution of the CO_2 from such compounds. The flask is now stoppered, and the solution is cooled to below 15°.

Flask B is now freed of atmospheric CO_2 by passing through it 250 ml of CO_2-free air. After the first 100 ml have been run through, standard $Ba(OH)_2$ solution is pipetted into the flask as follows: for the macroanalysis 3 ml of 0.25*N*, for the micro- 1.000 ml of 0.125*N*, and for the submicroanalysis 1.000 ml of 0.0155*N* hydroxide. The stream of CO_2-free air is continued through the flask while the $Ba(OH)_2$ is being pipetted in.

Into the amino-acid solution in flask A there are now introduced 50, 100, or 150 mg of ninhydrin with a glass spoon according to the size of the sample. Both flasks are quickly connected with the U-tube as shown in Fig. 1, the lower ends of the rubber connecting tubes being first dipped in water for lubrication. The apparatus is immediately evacuated with a water pump, and the clamp at the top is closed. Several analyses may be prepared as far as this stage, and then boiled together.

The entire apparatus is now immersed upright as far as the clamp in a

bath of boiling water for the time necessary to complete the reaction with ninhydrin. The times required are given in Table 1.

Table 1. Boiling Time for Completion of the Reaction with Varying Concentrations of Ninhydrin *

Ninhydrin Sample Volumes (ml)	50 mg		100 mg		150 mg	
	pH 2.5 (min)	pH 4.7 (min)	pH 2.5 (min)	pH 4.7 (min)	pH 2.5 (min)	pH 4.7 (min)
1	7	6	—	—	—	—
2	14	11	7.5	6	—	—
3	20	15	12	8	7	6
4	25	20	16	10	9	8
5	32	25	20	12	13	10

Distillation of CO_2. The distillation of the CO_2, with most of the water, from A into B is accomplished simply by lifting B over the edge of the hot water bath and immersing the lower half of B in cold water, while A and the limb of the U-tube above A remain in the boiling water. The time used to complete the distillation of CO_2 into B is 2 minutes when the volume of amino-acid solution in A is 1 or 2 ml; 3 minutes when it is 3 to 5 ml. The receiving flask is shaken during the distillation to mix the distillate with the $Ba(OH)_2$ solution. This is required to obtain complete CO_2 absorption.

When the distillation is finished, the apparatus is cooled and CO_2-free air from the reservoir is admitted through *D*. *D* is then closed again with the clamp and the apparatus is left connected until one is ready for the titration.

Titration. Macroanalysis. The titration is carried out with the *N*/7 HCl from a 5-ml burette. One drop of phenolphthalein solution is added as indicator. No especial precautions against atmospheric CO_2 are required, except that the titration is carried out at once after flask B is disconnected.

Microanalysis. This is done with *N*/35 HCl and with the 5-ml burette in the same manner as in the macroanalysis.

Submicroanalysis. The titration is carried out with *N*/14 HCl and the Rehberg micro burette. Immediately after disconnecting from the U-tube, the flask is placed on the stand of the Rehberg burette and a stream of CO_2-free air is started bubbling through the $Ba(OH)_2$ solution as rapidly as it can go without splashing the solution up on the walls of the flask. A drop of cresol red solution is added, and the acid from the burette is run in from the submerged tip until the color of the titrated solution matches that of an equal volume of veronal buffer solution containing 1 drop of cresol red.

Blank Analyses. With each series of micro- or submicrotitrations, duplicate blank analyses are performed the same day. The blank analysis is performed with an equal volume of water in place of the amino-acid solution, and with all the reagents used in the analysis except the ninhydrin.

* From Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).

Ninhydrin evolves no CO_2 from itself, and one can safely economize on the expensive reagent by omitting it from the blanks. The volume, T_1, of the standard HCl required in the blank analysis to neutralize the $Ba(OH)_2$ after the distillation, serves as the basis of the calculation in the analyses of amino acids. It is not necessary to determine the traces of CO_2 yielded by the water and reagents in the blank. The T_1 value includes a correction for these, as well as representing a standardization of the $Ba(OH)_2$ solution. For the macroanalyses the blanks need not be repeated for every series.

Calculations. If T_1 represents the ml of $N/7$, $N/14$, or $N/35$ HCl used in titrating the blank, and T_2 the ml of the same HCl used in the amino-acid analysis, the results are calculated as:

$$\text{Mg carboxyl nitrogen or carboxyl carbon} = (T_1 - T_2) \times \text{factor} \qquad (1)$$

For carboxyl nitrogen the factor is 1, when $N/7$ HCl is used; 0.2 when $N/35$ HCl is used; 0.5 when the HCl is $N/14$.

For carboxyl carbon the corresponding factors are 0.857, 0.1715, and 0.4285, respectively.

Unfortunately, according to Hamilton and Van Slyke,[11] the titration method described here cannot be applied to blood filtrates because traces of volatile acids distill with the CO_2 and cause positive errors. If picric acid filtrates are used, some of the picric acid also distills over. In the titrametric procedure of Christensen, West, and Dimick, the evolved CO_2 is bubbled through water before reaching the barium hydroxide. This treatment prevents the error from volatile acids, but the method does not yield precise results with the small amount of amino acids in 1 ml of plasma.

Remarks on Ninhydrin-Manometric Method for Amino Acids in Blood and Urine. The manometric method has been applied to blood plasma by MacFadyen[10] without any preliminary deproteinization or removal of urea. These substances evolve small amounts of CO_2, but corrections for them can be accurately applied. MacFadyen observed that clotting of the blood causes a 10 to 40 per cent increase in the amino-acid content of the resulting serum over that in the corresponding plasma.

Hamilton and Van Slyke[11] found that the amino acids could be most accurately determined on protein-free filtrates of plasma, unclotted whole blood, or erythrocytes deproteinized with picric acid.

A preliminary removal of urea with urease is required only on uremic blood. In non-uremic blood, the combination which takes place between urea and ninhydrin retards the hydrolysis of urea to such an extent as to reduce the CO_2 formed in this manner to a negligible quantity.

When the manometric method is applied to urine,[9b] the urea is first hydrolyzed with urease.

Titration of Amino Acids

Formol Titration. The best known of the titration methods for amino acids is the formol titration, first introduced by Sörenson.[14] In modern

chemical terms the reaction that takes place can be represented by the equation:

$$^{-}OOC \cdot R \cdot NH_3^{+} + CH_2O \longrightarrow {}^{-}OOCRNH_2 \cdot CH_2O + H^{+}$$

The formol titration has found extensive use in the investigations of protein chemistry. The gist of the procedure is to neutralize excess acidity in the solution to be titrated by a preliminary adjustment of the pH to between 6 and 7, add neutralized formaldehyde, and then titrate the solution with standardized alkali to about pH 9.0. According to Levy,[15] the maximum accuracy is obtained when, (1) the concentration of formaldehyde is maintained between 6 and 9 per cent, (2) the concentration of amino acids is as high as possible, (3) the end point of the titration is at pH 9.1, and (4) no correction is applied for a blank.

Indicators, neutral red for the preliminary neutralization to pH 7 and phenolphthalein for the final titration to pH 9 may be employed,[16] or the end points may be determined more accurately by means of the glass electrode.[17]

Van Slyke and Kirk [18] point out that the effect of preliminary neutralization of amino-acid mixtures changes the titration from an approximate measure of the carboxyl groups to an approximate measure of the primary amino groups plus the imino groups of the proline and hydroxyproline, together with any ammonia and free amines that may be present. The results are not sharply stoichiometric in all cases. When the titration is carried out to pH 9.0 as the end point, the titrations of proline and hydroxyproline are only about 80 per cent complete and of histidine about 88 per cent complete.

Dunn and Loshakoff [17] made use of the formol titration to determine the purity of amino acids. Using the glass electrode to determine the end point of the titration, monoaminomonocarboxylic acids could be determined with a precision of $\pm$ 0.1 per cent. The formol titration has been applied to the determination of the amino-acid content of the blood and urine.[18] Ultra-microanalytic procedures have been developed by Borsook and Dubnoff [19] and by Cisco, Cunningham, and Kirk.[20] These are applicable to between 5 and 15 γ of amino nitrogen.

Formaldehyde Titration of Blood and Urine.[18] *Blood.* On blood, the titration is carried out on a colloidal iron filtrate. Five ml of whole blood (no anticoagulant) is diluted with 35 ml of distilled water and heated to boiling in an Erlenmeyer flask. One ml of a 10 per cent solution of colloidal iron oxide is added, a few drops at a time, and the mixture is shaken after each addition. The mixture is transferred to a Pyrex test tube, made up with boiling water to a volume of 52 ml (= 50 ml at 20°), and immediately filtered. After cooling to room temperature, four 15-ml portions of the filtrate, each equivalent to 1.5 ml of blood, are evaporated to dryness and the residues are each dissolved in 2 ml of water. Two of the filtrates are used to prepare color standards for the starting point (pH 7 with neutral

red) and the end point (pH 9 with phenolphthalein). The other two blood-filtrates are titrated as follows: 1 drop of neutral red is added and the solution is titrated to match the neutral red standard. Then 0.4 ml of neutralized formalin and 3 drops of 0.1 per cent phenolphthalein are added and the solution is titrated with 0.01*N* NaOH to match the phenolphthalein standard.

Neutral red standard. This is prepared by adding 0.4 ml of 0.05*M* NaH_2PO_4 solution and 1 drop of neutral red to one of the filtrates. The solution is then titrated with alkali until it is at the point of the sharp color change of the indicator.

Phenolphthalein standard. One drop of neutral red, 1 drop of 0.1 per cent phenolphthalein, and 0.4 ml of about 40 per cent formaldehyde solution are added to a filtrate and 0.1*M* NaOH is added until the maximum color is developed.

From the volume of 0.01*N* NaOH used to titrate the blood filtrates, a correction is subtracted for the volume required to neutralize the amount of formaldehyde added. The calculation may be made as follows:

$$\text{Amino N in sample} = 0.14\ A \tag{2}$$

$$\text{Amino N per 100 ml blood} = 9.34\ A \tag{3}$$

where A represents the corrected number of ml of 0.01*N* NaOH used in the final titration.

Urine.[18, 21] Titration of urine requires the preliminary precipitation of albumin, if any is present, and of phosphate. The proteins of nephritic urine are precipitated by adding 5 drops of 10 per cent acetic acid to 50 ml of urine and then heating on a steam bath. The protein is filtered off and an equivalent of 50 ml of urine is introduced into a 100-ml volumetric flask. To this 2 gm of $BaCl_2$ is added and dissolved by shaking. The solution is then made alkaline to litmus paper by the addition of saturated $Ba(OH)_2$. The solution is now made up to 100 ml with distilled water, allowed to stand for 15 minutes, and then filtered. Fifty ml of the urine filtrate is concentrated *in vacuo* until the ammonia is removed. The residual solution is then acidified to litmus paper with 1*N* HCl and further distilled to remove CO_2. The sample is then transferred to a 50-ml volumetric flask, neutralized approximately with CO_2-free 1*N* NaOH, and made up to volume. One ml of the final solution represents 0.5 ml of urine. A 2-ml sample is used for the preparation of the Northrop alkaline color standard. The neutral red color standard is prepared from 1 ml of the original untreated urine. (Addition of phosphate to the barium-treated urine would cause precipitation of phosphate.)

Titrations of aliquots of the urine-filtrates are carried out as described for the titration of blood filtrates.

Acetone Titration. The principle of acetone titration is that addition of acetone changes the pK values of the amino acids through the consequent

lowering of the dielectric constant of the solution. Acetone moves the pK_1 values up far enough to make this buffer group titratable with HCl. Linderström-Lang introduced the acetone titration for the estimation of amino acids. It was applied to the estimation of amino acids in blood filtrates by Zirm and Benedict.[22]

In the Zirm and Benedict titration, blood serum is deproteinized with colloidal iron as described above. The aqueous filtrate is adjusted to pH 3.9 before acetone is added. Acetone (30 ml per 2 ml sample) is added and the titration is carried out with alcoholic HCl to the end point of the indicator used in the preliminary adjustment of the pH. The indicator employed is a 0.1 per cent alcoholic solution of α-naphthyl red (benzoyl-azo-α-naphthylamine). The colors are matched against appropriate standards. The color of the final titration, because of the effect of the acetone on the pK of the indicator, is equivalent to that of a water solution of about pH 4.8.

The color standards are prepared in the following manner.

Standard I is made by diluting 1 ml of 0.025*N* HCl to 400 ml with water. Two ml of this solution is mixed with 4 drops of a 0.1 per cent alcoholic solution of naphthyl red. The pH of the standard is approximately 3.9.

Standard II is prepared by mixing 2 ml of water, 0.3 ml of 0.025*N* HCl, 4 drops of the naphthyl red solution, and 30 ml of acetone.

Glacial Acetic Acid. Amino acids dissolved in glacial acetic acid solution are strong enough bases to be titrated with standardized solutions of perchloric, sulfuric, or hydrobromic acids.[23, 24] The end points may be determined electrometrically, or by indicators such as crystal violet or benzoyl auramine. Commonly 0.1 to 0.2 gm of amino acid is titrated in about 30 ml of glacial acetic acid. Arginine and lysine behave as diacidic bases, all others as monoacidic bases. The chemistry of the titration may be represented by the following equations:

$$(1)\quad {}^{+}NH_3 \cdot R{-}COO^{-} + HAc = {}^{+}NH_3 \cdot R{-}COOH + Ac^{-}$$
$$(2)\quad Ac^{-} + H^{+} = HAc$$

Clinical Significance of Amino-Acid Content of Blood and Urine

Protein metabolism is essentially the metabolism of the amino acids.[25, 26] The food proteins are hydrolyzed to amino acids during digestion. The proteins of the tissues are synthesized from the amino acids of the ingested foods and they in turn are hydrolyzed to amino acids when the tissues undergo autolysis. The amino acids also serve as the source of essential non-protein substances such as the purines, creatine, glutathione, carnosine, and the hormones thyroxine and epinephrine. Only a small fraction of the amino acids from the food is incorporated into the proteins of the body. The greater proportion is deaminized and the nitrogen-free residues may be converted into carbohydrate and fat or may be burned for body fuels.

The ingested proteins probably are completely hydrolyzed to amino acids by the proteolytic enzymes of the alimentary tract and the liberated amino acids are absorbed into the blood stream. During digestion, the amino-acid content of the blood may increase as much as 20 per cent. Of the absorbed amino acids, a large fraction is taken up and catabolized by the liver, the nitrogen being converted to urea. The tissues, as well as the blood, accumulate free amino acids. Even in the fasting animal the amino-acid concentration is about 10 times as great in the tissues as in the blood plasma; *i.e.*, about 40 to 60 mg of amino-acid nitrogen per 100 gm of fresh tissue as compared with about 5 mg per 100 ml of plasma. When amino acids are injected into the circulation, they are quickly taken up by the tissues. This increases the tissue amino-acid content 2 or 3-fold over the fasting level.[26]

Blood amino acids. The amino-acid concentration of the blood remains exceedingly constant. Most physiological and pathological changes induce little alteration in the blood amino-acid concentration.[27] Normal levels of blood amino-acid nitrogen determined by certain of the analytical methods are recorded in Table 2.

Table 2. The Amino-Acid Nitrogen Content of Normal Human Blood as Determined by Different Procedures

Ninhydrin Carbon Dioxide Method		Blood Plasma Nitrous Acid Method		Folin Colorimetric Method	
Range	Mean	Range	Mean	Range	Mean
3.84–5.52	4.36 ± 0.48 [11] *	3.78–5.16	4.40 [11]	4.8–7.8	6.3 [27]
3.35–5.00	4.07 ± 0.76 [10]				
2.3–6.6	4.3 ± 1.0 [11a]				
		Blood Corpuscles			
6.98–9.64	7.65 [11]	8.90–13.13	10.88 [11]		

The blood amino-acid nitrogen is not reduced even by prolonged fasting. The level is not significantly affected by age, by sex, or by normal pregnancy.[26] Certain endocrines have a noticeable effect on the blood amino-acid level. Insulin and epinephrine both have been found to lower the amino-acid nitrogen in spite of their opposite effects on the blood sugar.[28-30] Intravenous administration of the growth factor of the anterior pituitary, as well as of pitressin, antuitrin S, adrenal cortical hormone, testosterone propionate, and thyroxine induced marked increases in the amino-acid content of the plasma.[28]

In normal conditions, the liver is the sole site of the deamination of amino acids absorbed from the intestine.[31] The amount of hepatic tissue is greatly in excess of the amount required, so that 90 per cent or more of this organ must be removed before the metabolism of the amino acids is

* Superior numbers refer to references at the end of the chapter.

significantly interfered with. The determination of amino-acid nitrogen has its greatest diagnostic value in conditions associated with acute widespread degeneration of the liver. Elevated blood amino-acid values are found in acute yellow atrophy, phosphorus, chloroform, and carbon tetrachloride poisoning, arsphenamine hepatitis, cinchophen poisoning, and in some cases of eclampsia. In these conditions the amino-acid nitrogen of the blood plasma is usually between 10 and 15 mg per 100 ml with occasional higher values.[32] The increased concentration of amino acids in the blood is associated with a corresponding increase in the amino-acid content of the urine. Normally the content of the free amino acids of the urine is too low to permit their being isolated. In extensive hepatic degeneration, leucine and tyrosine may crystallize from the urine. The positive identification of the crystals of these amino acids is assumed to indicate extensive hepatic damage. The amino-acid level of the blood is of no value in the diagnosis of cases of incipient or advanced cases of liver disease without massive degeneration.[26]

Elevated levels of blood amino-acid nitrogen in eclampsia are presumed to be due to hepatic lesions which may occur in this condition.

Elevated blood amino-acid nitrogen is occasionally observed in advanced nephritis. In most cases of nephritis with high blood non-protein-nitrogen values, the amino-acid content remains within normal limits.[26]

Farr and MacFadyen [33] have observed that the plasma amino-acid concentration is subnormal in children suffering from nephrosis. They found that nephrotic crises were ushered in with a sudden further fall in plasma amino-acid concentration. This returned to the precritical subnormal level upon recovery.

Determination of the amino-acid content of the blood is of value in the control of intravenous amino-acid therapy.

Amino Acids in the Urine. The quantity of free amino acids normally excreted into the urine is slight. The amount ranges from 0.1 to 0.2 gm of amino-acid nitrogen daily, or 0.5 to 1.5 per cent of the total urinary nitrogen.[32] Considerable quantities of conjugated amino acids, in man principally glycine combined in the form of hippuric acid, may occur in the urine. The total amino-acid nitrogen of the urine is between 0.4 and 1.0 gm daily or about 2 to 6 per cent of the total urinary nitrogen.[32]

An increase in the free amino-acid content of the urine occurs in diseases with extensively impaired hepatic function or in conditions characterized by extensive tissue autolysis.[32]

In normal pregnancy, histidine becomes detectable in the urine at about the fifth week of gestation and disappears from the urine a few days after delivery. In pregnancy with eclampsia, the histidine disappears from the urine.[34-36]

Excretion of certain free amino acids in the urine occurs in several rare diseases that represent inborn errors of metabolism.[37]

Estimation of the Individual Amino Acids

Progress in the chemistry and physiology of the proteins is greatly aided by accurate and simple methods for the estimation of each of the known amino acids. This is a goal which is far from having been attained. In a recent review, Vickery [39] has classified the amino acids according to the accuracy with which they can be determined. His classification is reproduced in Table 3. Vickery originally listed 9 amino acids for which there are satisfactory methods of analysis. To his list there have been added serine, threonine, and hydroxylysine. Satisfactory methods for the determination of these amino acids have been recently developed, based on the reaction between α-hydroxyamino acids and periodate, introduced by Nicolet and Shinn.[40]

It would be beyond the scope of this work to present a detailed account of the different methods of analysis for each of the amino acids. The discussion will be confined to a consideration of the chemical principles

Table 3. Amino Acids Classified According to Degree of Accuracy with Which They Can Be Determined *

A. Amino acids concerning which our information is little better than qualitative.

Hydroxyglutamic acid	Isoleucine
Valine	Norleucine
Thyroxine	Diiodotyrosine

B. Amino acids for which methods of a considerable degree of probable accuracy have been proposed. These methods have been applied to very few proteins as yet.

Glycine	Proline
Alanine	Hydroxyproline
Leucine	Phenylalanine

C. Amino acids for which existing methods appear to give satisfactory results.

Cystine	Glutamic acid
Tyrosine	Arginine
Tryptophane	Histidine
Methionine	Lysine
Aspartic acid	Serine
Threonine	Hydroxylysine

which form the basis for the estimation of the amino acids that can be easily determined. Detailed descriptions of the procedures for the analysis of the amino acids are given in the monograph of Block and Bolling.[41]

Methods of Fractionation into Groups

For the determination of the amino-acid content of a protein, it is necessary to effect its complete hydrolysis, a detailed description of which is found in Chapter IV.

If a complete analysis is to be attempted, it is convenient first to separate the amino-acid hydrolysate into a number of fractions. Several procedures are available by which this can be carried out. The earliest of these is

* From Vickery, H. B., *Annals N. Y. Acad. Sci.*, **41**, 87 (1941).

steam distillation of the amino-acid esters. This method, developed by Fischer,[43] has fallen into disuse.

Dakin's Butyl Alcohol Method. This, which is probably the best of the methods, depends on the distribution of the amino acids between two phases: water saturated with butyl alcohol, and butyl alcohol saturated with water.[44] When a concentrated protein hydrolysate freed from acid is continuously extracted with butyl alcohol, the following fractions are obtained:

(1) The monoaminomonocarboxylic acids, both aliphatic and aromatic, insoluble in ethyl alcohol, but extracted with butyl alcohol.

(2) Proline, soluble in absolute ethyl alcohol and extracted with butyl alcohol. Hydroxyproline is extracted at atmospheric pressures, but not at reduced pressures. Propyl alcohol may be used to extract hydroxyproline under reduced pressure.

(3) Peptide anhydrides (diketopiperazines) are extracted by butyl alcohol, but are separated from the other extracted material by their sparing solubility in alcohol or water.

(4) The dicarboxylic acids are not extracted by butyl alcohol. They can be isolated as their calcium salts, which are insoluble in 80 per cent ethyl alcohol.

(5) The basic amino acids are not extracted by butyl alcohol and can be isolated by precipitation with phosphotungstic acid.

The Electrical Transport Method. The migration of the electrically charged amino acids in the field of a direct current can be used to make a partial fractionation of a protein hydrolysate.[45, 36] A protein hydrolysate, maintained at pH 5.5, separates into the following 3 fractions under the influence of a direct current; (1) the acidic amino acids, glutamic and aspartic, migrate toward the anode; (2) the basic amino acids, arginine, histidine, and lysine, migrate toward the cathode; and (3) the monoamino-monocarboxylic acids remain in the center compartment.

Copper Salts. The differential solubilities of the copper salts of the amino acids in water and in methyl alcohol was made the basis of a method for fractionation of amino acids by Schryver and his pupils.[47] The success of the method depends upon the use of very pure dry solvents and thoroughly dry copper salts. The copper salts of the amino acids are dehydrated with absolute acetone. They are then fractionated as follows:

I. Water-insoluble Fraction

It contains the copper salts of leucine, phenylalanine, and aspartic acid.

II. Water-soluble Fraction

It is fractionated with methyl alcohol into:

(a) Copper salts which are insoluble in methyl alcohol.

This fraction contains alanine, tyrosine, glycine, lysine, arginine, histidine, and glutamic acid.

(b) Copper salts which are soluble in methyl alcohol. This fraction contains valine, hydroxyvaline, proline, and prolylphenylalanine.

New General Quantitative Methods

Certain new general principles have been suggested for the quantitative estimation of amino acids. Two new chemical developments are the solubility method of Bergmann and Stein [48] and the isotope dilution method of Rittenberg and Foster.[49] They have in common the premise that it is impossible quantitatively to isolate every component of a mixture such as a protein hydrolysate. Both methods attempt a quantitative gravimetric analysis without the necessity of quantitatively isolating the substance subjected to analysis.

The solubility method is based upon the physical chemical principle that the solubility product of the ions of a saturated solution is a constant. The application of the solubility method to the determination of amino acids is shown graphically in Fig. 2, from which it will be noted that the solubility products, K_1 and K_2, do not appear in the final equation. Thus the final answer is, within considerable limits, independent of the absolute value of the solubility of the amino-acid salt.

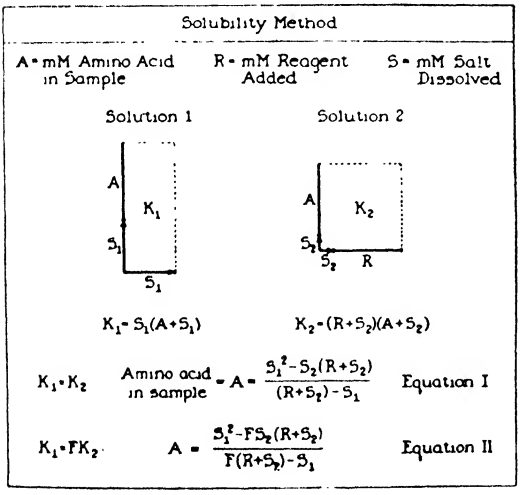


FIG. 2. The solubility method applied under conditions in which the solid phase is preformed and allowed to dissolve in the sample instead of being formed by precipitation from the sample. R represents that portion of the sulfonic acid added as such. S represents the sulfonic acid contributed to the solution by the salt as it dissolves.

From Moore, S., Stein, W. H., and Bergmann, M., *Chem. Rev.*, **30**, 423 (1942).

The development of the solubility method has simplified the search for reagents useful for analysis of the amino acids. It is no longer necessary to seek reagents that quantitatively and selectively precipitate only one amino acid. Amino-acid salts with a solubility in water at 0° of the order of 1 to 5 per cent have been found to be most useful for analysis by the new method.

In applying the solubility method, the aromatic sulfonic acids have been found to be particularly useful reagents. Besides forming sparingly

soluble salts with amino acids, the sulfonic acids are almost as strong acids as hydrochloric or sulfuric acid. Consequently they will form dissociable salts with any substances possessing a basic group. This property considerably simplifies the application of the solubility product principle. Some examples of useful sulfonic acids are: 2-bromotoluene-5-sulfonic acid for the estimation of leucine, 2,5-dichlorobenzene sulfonic acid for phenylalanine, etc.

The isotope dilution method makes use of the principle that if a known amount of an isotopically labeled amino acid is added to a mixture, and then some of the amino acid is isolated, the percentage of isotope in the isolated amino acid will bear a quantitative proportionality to the total amount of the amino acid in the mixture. The quantity can be calculated from the equation

$$y = \left(\frac{Co}{C} - 1\right) x \tag{4}$$

where y is the quantity of amino acid in the mixture, x is amount of labeled amino acid added, Co is the isotope concentration above normal of the amino acid added, and C is the isotope content of the isolated amino acids.

At present this tool is restricted because of the limitations that exist for the isolation and measurement of isotopes. In the work carried out by Rittenberg and Foster, heavy nitrogen, N^{15}, was used to label the amino acids. In some instances deuterium, which is much easier to obtain and to estimate, has been employed for this purpose. For the sulfur-containing amino acids, S^{35} could be similarly used.

Microbiological Assay. Biological methods for the estimation of amino acids that appear to hold great promise are assays with the lactic acid producing organisms, *lactobacillus arabinosus*, and *lactobacillus casei*,[50, 51] and with mutants of the ascomycete *Neurospora crassa*.[52]

The growth and lactic-acid production of the *lactobacillus* organisms are functions of certain essential nutrilites. These include some of the amino acids. By preparing media in which only one of the essential amino acids for the microorganism is made the limiting factor, the growth, and more particularly the amount of lactic acid formed, can be used to estimate the content of the amino acid. By this procedure it is now possible to carry out assays for arginine, glutamic acid, leucine, isoleucine, phenylalanine, tryptophane, tyrosine, and valine. Kuiken and co-workers [51] have determined the valine, leucine, and isoleucine content of a number of proteins by this method. The method appears to be accurate to within 2 per cent.

Developments with the *Neurospora* have not reached such a concrete stage. In the case of this organism, x-ray or ultraviolet irradiation gives rise to biochemical mutants that require growth factors not needed by the untreated organism. Certain of the mutants that have been produced have lost their ability to synthesize specific amino acids, *e.g.*, isoleucine and

valine. It seems probable that measurement of the growth rate of the mutants can be employed for the biological determination of a number of the amino acids.

Special Methods for the Estimation of Individual Amino Acids

Arginine, Lysine, and Histidine. Satisfactory methods exist for the determination of arginine and histidine; the methods for lysine, however, are not on the same level of accuracy. A standard method for the isolation of the 3 basic amino acids depends on the differential precipitation of their respective silver salts from protein hydrolyzates.[53, 54] The silver salts of histidine and arginine are precipitated from a hydrolyzate at pH 9–12. The lysine is left behind and may be isolated as the phosphotungstate. The precipitate of silver arginine and histidine is redissolved and the silver histidine salt is precipitated by adjusting the pH to exactly 7.4, leaving the arginine in solution.

The above 3 amino acids can also be determined in the phosphotungstic acid precipitate from a protein hydrolyzate by the nitrogen distribution analysis of Van Slyke.[55] This method depends upon the facts that one-half of the nitrogen of arginine (of the guanidine group) is hydrolyzed to ammonia by heating with concentrated alkali and that in the reaction with nitrous acid, arginine yields only $\frac{1}{4}$ and histidine $\frac{1}{3}$ of its nitrogen. It has long been realized that the Van Slyke nitrogen-distribution method is not capable of giving results of more than semi-quantitative accuracy.

More satisfactory, particularly for arginine and histidine, are methods based on certain specific properties of these amino acids. Arginine is quantitatively precipitated by flavianic acid (1-naphthol-2,4-dinitro-7-sulfonic acid). Vickery [56] has developed a modification of this procedure which he states is definitely superior to other methods on grounds of accuracy and precision.

The specific action of the enzyme arginase from mammalian liver in hydrolyzing arginase to ornithine and urea forms the basis of an accurate quantitative method for the estimation of this amino acid.[57, 58]

The most widely used procedures for determining arginine, however, are based on the Sakaguchi color reaction.[59] In this reaction, an alkaline solution of arginine, because of its guanidino group, yields a red color when it is treated with α-naphthol and an oxidizing agent such as hypobromite.

The color reaction suffers from a number of drawbacks as the basis of a quantitative analytical method. The color fades rapidly due to the destruction of the colored compound by excess hypobromite. To retard fading, Weber [60] adopted the expedient of adding urea to stabilize the color by reacting with the excess of hypobromite. Another drawback to the Sakaguchi method is that many substances, in particular ammonia, creatine, tyrosine, tryptophane, and histidine, inhibit the color production.

Many modifications of the color reaction have been introduced in efforts

to improve the specificity and accuracy of the determination of arginine.[60-65] MacPherson [64] found that the color was stabilized by adding urea prior to the addition of hypobromite. Dubnoff,[63] in a micromethod designed for biological material, first separates the arginine by adsorption on Permutit to eliminate interfering substances, and then carries out the color reaction after eluting the arginine from the Permutit with 3 per cent NaCl solution.

Brand and Kassel [65] carry out the determination on a series of dilutions of a protein hydrolysate and extrapolate the arginine values to zero arginine concentration to obtain correct results. This scheme is based on the observation [61] that the color intensity decreases as a linear function of the increase in the amount of arginine employed for the analysis.

The Kapeller-Adler color reaction [66] is widely used in the estimation of histidine. In this reaction, a blue-violet color is obtained when an alkaline solution of histidine is brominated or is treated with diazotized sulfanilic acid. The histidine reaction suffers from the same drawbacks as the arginine reaction, namely, a rapid fading of the color and interference by many substances with the color development. Many modifications have been suggested to obviate this difficulty.[64, 67-69] In general, it is first necessary to precipitate histidine from protein hydrolysates with $AgNO_3$ and $HgSO_4$ in 5 per cent H_2SO_4. Woolley and Peterson [70] recommend that the histidine be precipitated with phosphotungstic acid as well as with silver and mercury before applying the Kapeller-Adler reaction.

Tryptophane, Phenylalanine, Tyrosine, Dihydroxyphenylalanine, Diiodotyrosine, and Thyroxine. Although there are separate methods for the estimation of tryptophane, both it and tyrosine are generally determined according to the same scheme.

Independent methods for tryptophane are those based on its ability to couple with aldehydes in the presence of a condensing agent. In the Hopkins-Cole test, tryptophane reacts with glyoxylic acid in the presence of concentrated sulfuric acid to give a blue-violet color. This is commonly used as a qualitative test for proteins. It depends upon the presence of tryptophane. The method can also be used for the quantitative determination of tryptophane. The aldehyde, *p*-dimethylaminobenzaldehyde (Ehrlich's reagent), is more commonly used than glyoxylic acid for this purpose. When a compound containing the indole nucleus, in the case of proteins one containing tryptophane, is treated with Ehrlich's reagent in the presence of concentrated HCl, a dark-blue color develops, which is quite stable and can be used for the quantitative determination of tryptophane.[71-73] Addition of a suitable accelerator (oxidizing agent) increases the speed of the color development so that maximum color formation is obtained in 5 to 10 minutes. Sodium nitrite is used as an accelerating agent by Bates.[72] With the aldehyde reaction, it is not necessary to hydrolyze a protein to secure the free tryptophane. The reaction is given by con-

jugated tryptophane, but the shade of color may vary with the nature of the groups united to this amino acid.

Phenylalanine may be determined by nitrating it to 3,4-dinitrophenylalanine. The nitro compound is then reduced with hydroxylamine in the presence of ammonia. The purple color of the resulting ammonium 3,4-dihydronitrophenylalaninate is estimated colorimetrically.[74, 75]

Tyrosine and tryptophane are more commonly determined by modifications of the method of Folin and Ciocalteau.[76-79] In this procedure the protein is hydrolyzed with alkali and tryptophane is removed with the Hopkins Cole reagent (15 per cent $HgSO_4$ in $6N$ H_2SO_4). The H_2SO_4 at a concentration of 3.5 to 7.5 per cent prevents the precipitation of tyrosine. The tyrosine is then estimated colorimetrically either with the phenol reagent of Folin and Ciocalteau or by means of Millon's reagent. The phenol reagent is a phosphotungstic-phosphomolybdic acid stabilized with lithium sulfate, which is reduced by phenols to yield an intense blue color. Ordinarily color development with Millon's reagent is slow, but upon boiling tyrosine with $HgSO_4$, it becomes capable of reacting almost instantly with either Millon's reagent or simply with sodium nitrite to yield an orange color.

The precipitated tryptophane is freed from mercury, and determined by the color it develops with the phenol reagent. Brand and Kassell[79] have adapted the above procedures to photometric determination with the Pulfrich photometer.

By taking advantage of the fact that hydrolysis with alkaline stannite solution converts diiodotyrosine and thyroxine to reactive phenols, the above authors determine these amino acids with Millon's reagent.

Dihydroxyphenylalanine (dopa) can be estimated by treatment with nitrous and phosphomolybdic acids. This gives a yellow-colored substance which changes to orange-red in alkaline solution.[80]

The Sulfur-containing Amino Acids: Cysteine, Cystine, Methionine

A system of analysis for the sulfur-containing amino acids has been devised by Baernstein.[81] In this procedure, the protein is hydrolyzed with hydriodic acid. Hydrolysis with this acid is more rapid; humin is not formed and reactive fragments of carbohydrate are eliminated. Cystine is reduced to cysteine, methionine is demethylated with the formation of thiolactone, and sulfates are reduced.

In this system of analysis, the cysteine from cystine is determined colorimetrically by the method of Sullivan[82] or titrametrically by the Okuda method.[83] The methionine is determined either by estimation of the volatile methyl iodide which is formed, or by titration of the resulting homocysteine with tetrathionate, after opening the thiolactone ring with alkali.

Lavine[84] has proposed a method for the estimation of methionine based

on its reversible reaction with iodine. Thio-ethers like methionine form perhalides according to the reaction

$$R_2S + X_2 \rightleftharpoons R_2S \cdot X_2$$

Acidification of the solution results in the reversal of the above reaction with the liberation of I_2, which may be titrated with thiosulfate. By carrying out the iodination reaction at pH 7 in 1*M* KI solution, a considerable specificity for methionine is achieved. McCarthy and Sullivan [85] suggest the use of the red color which is developed when methionine is treated with sodium nitroprusside in alkaline solution and then is acidified, for the estimation of methionine.

A number of color reactions have been discovered which are quite specific for cysteine. These are used to determine cysteine, and after reduction, cystine.

The Sullivan reaction depends upon the color developed when cysteine reacts with 1,2-naphthoquinone-4-sulfonate in a highly alkaline solution. The red color which develops with cysteine, unlike that with other amino acids, is not discharged by reducing agents such as sodium hyposulfite ($Na_2S_2O_4$). The reaction has been studied and modified by a number of investigators.[86, 87] Zittle and O'Dell [88] found that cystine is reduced and precipitated quantitatively by cuprous oxide in acid solution. The cysteine in the mercaptide precipitate is determined by the Sullivan reaction, after removal of the copper with thiocyanate.

Another colorimetric reaction for the estimation of cysteine and cystine is the blue color obtained when these amino acids are heated with *p*-aminodimethylaniline in acid solution in the presence of ferric ammonium sulfate. The formation of the typical blue color appears to require a thiol group and a primary amine separated from each other by two $—CH_2—$ groups, as is found in cystine and cysteine.[89]

In the Okuda method,[83] cystine is reduced to cysteine and the latter is titrated with a solution of potassium bromate in the presence of potassium iodide.

Hydroxy Amino Acids: Serine, Threonine, and Hydroxylysine

These three amino acids can be readily determined from the different aldehydes which are formed as a result of their oxidation with periodate. Nicolet and Shinn [40] adapted the Malaprade [90] reaction for compounds with adjacent hydroxy groups to the hydroxy amino acids. The reaction that takes place is illustrated for the amino acid threonine by the equation

$$CH_3—CHOH—CH(NH_2)COOH + IO_4^- = CH_3CHO + OHC—COOH + NH_3 + IO_3^-$$

The acetaldehyde formed from threonine is aerated into sodium bisulfate and determined iodometrically.[91] Winnick [92] has developed this into a micro diffusion method by treating the hydrolysate with neutral periodate

in the outer chamber of a Conway diffusion cell, and allowing the acetaldehyde which results to diffuse into bisulfate solution in the central chamber.

In the case of serine, the formaldehyde formed by the action of periodate is determined as the dimedon derivative.[93]

For the determination of hydroxylysine, this amino acid is precipitated from protein hydrolysates with the other dibasic amino acids by means of phosphotungstic acid. The hydroxylysine is estimated from the ammonia liberated by the reaction with periodate.[94]

The Dicarboxylic Amino Acids: Glutamic and Aspartic Acids*

The dicarboxylic amino acids are isolated because of the insolubility of their calcium salts in ethanol. This method, which was adopted by Foreman,[95] has been critically studied and improved by Chibnall and his co-workers.[96, 97] The latter workers developed a procedure in which no reagent was introduced that could not be quantitatively removed without any appreciable loss of nitrogen. Cystine is first removed as the cysteine cuprous mercaptide; otherwise sulfinic or sulfonic acids, which are formed during the treatment with $Ca(OH)_2$, are precipitated with the calcium dicarboxylates and interfere with the subsequent analysis. After the preliminary precipitation, the glutamic acid is usually isolated as the insoluble hydrochloride and the aspartic acid as the copper salt. If all precautions are taken, the two dicarboxylic acids can be determined with an accuracy of about 98 per cent, according to Chibnall and co-workers.

Aspartic acid can also be determined by deaminating it to malic acid and then brominating it to dibromomalic acid.[98] The latter is oxidized by $KMnO_4$ to dibromo-oxalacetic acid which can be isolated by steam distillation. The dibromo-oxalacetic acid combines with dinitrophenylhydrazine in acid solution, yielding a hydrazone which is insoluble in water. The compound is soluble in pyridine and yields a blue color on addition of alkali. The color is estimated in a photoelectric colorimeter.

Determination and Clinical Significance of the Plasma Proteins

Nature and Origin of the Plasma Proteins

Determination of the concentration of the plasma proteins offers an extremely valuable method for the differential diagnosis of a large number

* A number of methods of analysis for this group of amino acids have appeared since completion of the manuscript. In the procedure of Cannan [98a], the dicarboxylic amino acids are adsorbed from a protein hydrolysate by a basic Amberlite resin and eluted from the resin with HCl. The glutamic acid hydrochloride and copper aspartate may be crystallized directly in pure form from the eluate. The glutamic and aspartic acids in the eluate may be estimated by means of an electrometric titration in water and in formaldehyde solution.[98b] Olcott [98c] has developed a method for the determination of glutamic acid based upon a measurement of the loss in amino nitrogen resulting from the transformation of glutamic acid to pyrrolidonecarboxylic acid at 125° and pH 3.3.

of diseases and also a means of determining the effectiveness of various therapeutic measures. The metabolism of the amino acids and of the plasma proteins are most intimately related. When amino acids are administered, it is to correct a deficiency of the plasma proteins.[99-101]

Estimation of the individual plasma proteins is a difficult problem. Modern investigation with the Tiselius electrophoresis apparatus shows that normal plasma contains albumin, fibrinogen, and three other globulin components, designated as alpha, beta, and gamma globulin.[102, 103] The typical normal electrophoresis pattern is shown in Fig. 3. Chemical evidence indicates that there may be more protein components in the blood plasma than is given by the number of migrating boundaries in an electrical field.[104]

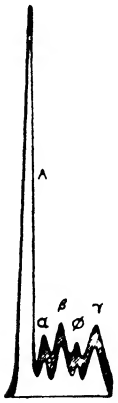


FIG. 3. The range of variation of components of twelve normal plasma patterns (shaded area). A = albumin; α = α-globulin; β = β-globulin; γ = γ-globulin; ϕ = fibrinogen. From Moore, D. H., and Lynn, J., *J. Biol. Chem.*, **141**, 819 (1941).

The origin of the plasma proteins is not a completely solved problem. The liver appears to be the primary site of synthesis.[105, 106] Fibrinogen production is probably wholly dependent on liver function.[106] Albumin and globulin are elaborated by the liver, but a growing body of evidence indicates that these two proteins can be formed by cells of the reticuloendothelial system outside the liver. The high content of serum globulin found in diseases involving bone marrow indicates that the bone marrow cells elaborate this group of proteins.[107]

The plasma proteins usually are synthesized from the amino acids of the foodstuffs. Parenteral injections of amino acids or of protein hydrolyzates can serve as a source of plasma protein nitrogen. This is a subject of

Table 4. Range of Concentration of Normal Plasma Proteins in Grams per 100 ml of Plasma *

	Total Protein	Albumin	Globulin	Albumin to Globulin Ratio	Fibrinogen
Usual	6.0–8.0	3.6–5.4	1.5–3.4	1.2–2.6	0.2–0.4
Extreme	5.6–8.4	3.4–5.6	1.35–3.55		

* From Myers, V. C., and Muntwyler, E., *Physiol. Rev.*, **20**, 1 (1940).

increasing interest in medical cases that do not tolerate oral ingestion of liquid or solid food.[99-101] In the opinion of Whipple and co-workers,[106] under certain conditions, cystine appears to serve as a key amino acid in determining the utilization of amino acids for the synthesis of the plasma proteins.

There is an intimate relationship between the plasma proteins and the tissue proteins, particularly the proteins of the liver. During protein deprivation, as in fasting, the plasma protein level appears to be maintained at the expense of the tissues, particularly the liver proteins.[108] Conversely, it is believed that the plasma proteins can serve as a source of protein storage material for the liver in the event of a plethora.

Protein Levels in Health and Disease

The normal range of values of the plasma proteins is given in Table 4. Formerly, considerable attention was paid to the fractionation of globulin into euglobulin and pseudoglobulin. Electrophoretic studies have shown that three globulin components are present normally; namely, α-, β-, and γ-globulin. This finding has obscured the significance of the pseudo- and euglobulin. No considerable amount of data is as yet available on the distribution of the 3 newly named globulin components in health and disease.

Upset of the plasma protein content is a very common accompaniment of disease. Alterations occur in many diseases. A list of diseases in which hypoproteinemia or hyperproteinemia may occur, as taken from the compilation of Kagan,[110] is given in Table 5.

Hypoproteinemia is more common clinically than is hyperproteinemia. When hypoproteinemia is present, it is invariably due to a depletion or loss of the serum albumin. Diseases of the kidney are the most important group in which hypoalbuminemia occurs. Hypoalbuminemia is also common in patients showing evidence of malnutrition.

The serum albumin is the most important protein in the maintenance of the colloid osmotic pressure of the blood. The most prominent clinical feature of a gross diminution in the colloid osmotic pressure is *edema*. The lack of albumin is chiefly responsible for the edema resulting from a hypoproteinemia. Because of its lower molecular weight and greater number of acid groups, serum albumin exerts a greater osmotic effect per gram than does globulin.

In *hyperproteinemia*, it is almost invariably the serum globulin that is increased. Important diseases in which there is hyperglobulinemia are multiple myeloma, lymphogranuloma inguinal,[111] subacute bacterial endocarditis, leprosy, and kala-azar.[110]

Increase in the fibrinogen content may occur in a variety of conditions, such as in slight hepatic injury, acute infections, pregnancy and menstrua-

Table 5. Pathological Conditions with Altered Serum Protein Concentration *

Hypoproteinemia Below 6.0 gm per 100 cc	Hyperproteinemia Above 8.0 gm per 100 cc
1. *Malnutrition* Dietary (a) Endemic and sporadic (b) Associated with chronic infection, pellagra, beri beri Poor Absorption (a) Diarrhea (b) Intestinal fistulae, ileostomy	1. *Dehydration* Insufficient intake Fluid loss (a) Intestinal obstruction and fistulae (b) Diarrhea, especially infants (c) Cholera (d) Diabetic acidosis (e) Vomiting (f) Burns (g) Heat exhaustion (h) Fulminant infections (i) Addison's disease
2. *Kidney Diseases* Nephroses — all types Glomerular nephritis, chronic Amyloid kidney	2. *Diseases Involving Bone Marrow* Multiple myeloma Malignant metastases to bone
3. *Liver Diseases* Cirrhosis Cancer	3. *Infections, Chronic* Syphilis, Trypanosomiasis Subacute bacterial endocarditis Lymphogranuloma inguinal Leprosy, Kala-azar Boeck's sarcoid Malaria, Filariasis
4. *Protein Dilution* Excess fluid administration Fever	4. *Liver Diseases* Cirrhosis Cancer, primary or metastatic
5. *Protein Loss* Hemorrhage Weeping wounds Shock	
6. *Heart Failure*	
7. *Hyperthyroidism*	
8. *Chronic Poisoning* Benzene, War gases	
9. *Toxemias of Pregnancy*	

tion, focal infections, nephrosis, and following x-ray irradiation.[32, 112] Decreased plasma fibrinogen is found in conditions of hepatic insufficiency, typhoid fever, and cachectic conditions, notably malignancy.

Considerable significance has been attached to alterations in the albumin: globulin ratio in clinical medicine. Kagan [110] points out that the use of the A/G ratio may be misleading. The ratio gives no true insight into actual change in either the albumin or globulin concentration.

Kagan points out that the A/G ratio is often reversed in lymphopathia venereum and in nephritis. However, in lymphopathia venereum, this is due to an increase in the globulin; in nephritis, it is due to a decrease in the albumin fraction. Therefore, it is the absolute values of the protein concentrations that should receive attention in diagnosis, and not the A/G ratio.

Determination of Total Serum Protein

Determination of the total serum protein content can be made more readily than that of the different protein components. In the majority of instances, the changes taking place in the circulatory system can be

* From Kagan, B. M., *Southern Med. J.*, **36**, 234 (1943).

tionship has been shown to exist between the index of refraction and the concentration of the serum proteins. The concentration of the protein may be evaluated from the equation

$$C = \frac{1}{\alpha}(N_1 - N_s) \qquad (5)^*$$

where C represents the protein concentration in grams per 100 cc of solution; α the specific refraction; N_1 the refractive index of the protein solution; and N_s the refractive index of the pure solvent. Little use is made of the refraction method at the present time.

Specific-gravity Methods. Methods for the determination of the serum protein concentration derived from the specific gravity of the blood serum or plasma have recently become very popular. The reason for this is their relative simplicity and extreme rapidity. Only a drop of blood is required and the determination can be carried out at the bedside.

That there exists a linear relationship between the protein content and the specific gravity was determined on nephrotic patients by Moore and Van Slyke.[114] For heparinized blood plasma they offered the formula:

$$\text{Total Protein} = 343\ (\text{sp. gr.} - 1.007) \qquad (6)$$

For blood serum there has been obtained the formula [115]

$$\text{Total Protein} = 345\ (\text{sp. gr.} - 1.0076) \qquad (7)$$

In the hands of Moore and Van Slyke, the specific gravity determination was carried out simply by weighing a known volume of blood serum in a pyknometer.

In the methods of Barbour and Hamilton [116] and of Kagan [115] the specific gravity is determined from the rate of fall of a drop of the blood serum or plasma through a viscous medium. Barbour and Hamilton employed a mixture of xylene and bromobenzene in a tube 30 cm long. The specific gravity is read off an alignment chart that relates falling time and temperature and the density difference between the drop and the fluid mixture. Calibrations are made with aqueous standards.

The Kagan method employs a mixture of methyl salicylate and liquid petrolatum. Calculations are made upon the principle of Stokes' law for the fall of a sphere through a viscous medium. This requires that the size of the drop be maintained constant. In the proteinometer of Kagan, the radius of the drop of blood serum is standardized by employing an exactly identical volume in each determination. Specific gravity of the serum is determined from tables based on the Stokes' equation

$$V = \frac{2}{9} g r^2 \frac{s - d}{\eta}$$

where V is the velocity of fall in cm per second, g the gravity constant, r the radius of the falling sphere, s the density of the falling sphere, d the density of the liquid, and η the viscosity of the liquid through which the

* A more accurate formula is given by Sunderman.[116a]

sphere falls. From the specific gravity so obtained, the protein concentration is evaluated from the numerical relations embodied in the formulas for plasma (6) and serum (7) given above.

It should be kept in mind that the protein content may not be the only factor that influences the specific gravity of the blood serum. It might reasonably be expected that in sera of cases with marked nitrogen retention or high in glucose, the calculated protein values would be in error. Looney [117] was unable to confirm the findings of those investigators [114, 115] who observed a high correlation between specific gravity and the serum-protein concentration. The sera of 10 normal persons and 14 schizophrenic patients showed a very low correlation coefficient between the specific gravity and the protein content. Similar discrepancies have been reported by other workers.[118, 119]

Turbidometric Method.[120] Total serum protein may be determined turbidometrically by diluting serum in the proportion of 1:10 with 1 per cent NaCl solution. To 2 ml of this dilution 0.5 ml of 2 per cent gum ghatti and 2.5 ml of 5 per cent sulfosalicylic acid are added. The resulting degree of turbidity in the solution is read in a photoelectric colorimeter and the total protein determined by comparison with a standard curve.

Colorimetric Methods. The determination of total serum protein by the biuret and the tyrosine color reactions is described in the next section.

Determination of Albumin and Globulin

Salting-out Procedures. The standard salt used for separating albumins and globulins is $(NH_4)_2SO_4$. A method of determining globulin and albumin in blood serum by salting out the globulin by half saturation with $(NH_4)_2SO_4$ was devised by Cullen and Van Slyke.[121] The $(NH_4)_2SO_4$ is removed by distillation with MgO, and the nitrogen of the protein residue is determined by the Kjeldahl method. It is readily seen that the presence of the ammonium ion is a great disadvantage.

To avoid the use of ammonium salt, Howe [122] employed sodium sulfate for salting out plasma proteins. This method is probably the one most widely used at present. Its one defect is that the temperature has to be maintained at about 37° to secure a sufficiently high degree of solubility of the Na_2SO_4.

The concentrations of Na_2SO_4 required to precipitate the different proteins of blood plasma are given in Table 6.[5b, p. 680]

After separating and washing the different protein fractions, each may be determined by the Kjeldahl nitrogen method. Values for albumin are obtained either by analyzing the globulin-free serum residue or by difference between total protein and total globulin.

Campbell and Hanna [123] have proposed the use of Na_2SO_3 in place of Na_2SO_4 to estimate the plasma proteins. They state that: "Determination of the albumin and globulin content of serum can be accomplished simply

Table 6 *

Protein	Na_2SO_4 Concentration Molar	Per Cent
Fibrinogen	0.76	10.6
Euglobulin	1.00	14.2
Pseudoglobulin I	1.25	17.7
Pseudoglobulin II	1.50	21.5

and rapidly by the use of sodium sulfite as a protein precipitant and a copper-selenium-phosphoric-sulfuric acid mixture as the protein digestant." Serum globulin is salted out by a 21 per cent Na_2SO_3 solution using 1 part of serum to 19 parts of the sulfite. A high temperature is not required to keep the Na_2SO_3 in solution.

Filtration through ordinary filter paper may cause a considerable loss of albumin because of adsorption. To minimize this it is recommended that hardened filter paper be used.[124]

After the components of the serum proteins are salted out, they may be determined by a variety of methods besides that of estimating the nitrogen content by the Kjeldahl procedure.

Turbidometric Method. In the method of Looney and Walsh,[120] the globulin is determined by diluting 1 ml of blood serum with 1 per cent saline to 10 ml. One ml of the diluted serum is mixed with 2 ml of 2 per cent gum ghatti, and then 3 ml of a saturated $(NH_4)_2SO_4$ solution are added. After mixing, the turbidity is measured in a photoelectric colorimeter. Comparison with a standard curve gives the globulin content. Albumin is obtained by difference between the total protein and the globulin content.

Colorimetric Methods. *The Biuret Reaction.* The application of the biuret color test for the quantitative determination of serum proteins was introduced by Autenrieth.[125] It has since undergone considerable modification. A careful study of this reaction has been carried out by Robinson and Hogden.[126] In their procedure, *total serum protein* is determined on 0.2 ml of serum by precipitating the protein with 10 per cent trichloroacetic acid, dissolving the precipitate with NaOH, adding 0.25 ml of 20 per cent $CuSO_4$, and then adjusting the volume to 10 ml. The color is compared in a photoelectric or a Duboscq colorimeter against standards prepared from known dilutions of a standardized blood serum. Serum albumin is determined by first salting out the globulin with 30 parts of 22 per cent Na_2SO_4, and precipitating the albumin in the filtrate with trichloroacetic acid. The rest of the procedure is the same as for total protein. When a photoelectric colorimeter is employed it is desirable to use a 560 mμ filter.

The Folin Phenol Reaction. This method, first introduced by Wu,[127] has also undergone a variety of modifications. The procedure of Greenberg [128] and of Greenberg and Mirolubova [129] is described here in detail.

* From Peters, J. P., and Van Slyke, D. D., "Quantitative Clinical Chemistry," Vol. II, Williams and Wilkins Co., Baltimore, Md., 1932.

Reagents: Na_2SO_4 Solution. 22.5 gm anhydrous Na_2SO_4 is dissolved in water to a volume of 100 ml. This solution must be kept at 38° to prevent crystallization.

5N NaOH Solution.

Tyrosine Standard. Dissolve 200 mg of pure tyrosine to 1 liter with approximately 0.1*N* HCl. Add 1 drop of 1:1000 merthiolate as a preservative.

Folin and Ciocalteau Phenol Reagent.[76] Dissolve 100 gm of sodium tungstate, $Na_2WO_4 \cdot 2\,H_2O$, and 25 gm of sodium molybdate, $Na_2MoO_4 \cdot 2\,H_2O$, in 700 ml of water in a 1500-ml Florence flask. Add 50 ml of syrupy (85 per cent) H_3PO_4 and 100 ml of concentrated HCl. Connect the flask with a reflux condenser by means of a cork or rubber stopper wrapped in tinfoil. Boil the solution gently for 10 hours. After boiling, add 150 gm of Li_2SO_4, 50 ml of H_2O and a few drops of liquid bromine. Boil without the condenser for about 15 minutes to remove the excess bromine. Cool, dilute to one liter, and filter. The finished reagent should be straw yellow in color. Keep in a glass-stoppered bottle.

Procedure. Total Protein. Dilute the serum in the proportion of 1 to 10 with 0.9 per cent NaCl. Pipette 2 ml of the diluted serum into a 20-ml test tube, and add about 5 ml of H_2O and 2 ml of 5*N* NaOH. Mix the contents and heat in a briskly boiling water bath for 10 minutes. Now insert a funnel into a 50-ml volumetric flask and transfer the contents of the tube into the flask, washing out the tube with several portions of distilled water. Add 3 ml of the phenol reagent, make up to the graduation mark and read the color against a standard containing 5 ml of the standard tyrosine solution.

Albumin and Globulin. Pipette 0.5 ml of serum or plasma from a calibrated pipette into a 20-ml test tube. Add exactly 9.5 ml of 22.5 per cent Na_2SO_4 solution with a pipette of that volume or from a burette. Stopper, mix thoroughly, and set aside for about 2 hours in an incubator at 37 to 38° to allow coagulation of the globulin. At the end of this period, filter into another test tube, using a fairly retentive filter paper (Whatman No. 42 is satisfactory). Examine to see that the filtrate is clear; if not, pour back onto the filter paper. After the filtration is nearly complete, remove the tube containing the filtrate to be used for albumin analysis. Five ml of the albumin filtrate are pipetted into a 20-ml test tube; then 5 ml of water and 2 ml of 5*N* NaOH are added. After mixing the contents, the tube is heated in the boiling water bath for 10 minutes. The rest of the procedure is the same as that given above for the total protein.

Globulin may be estimated by difference between total serum protein and albumin or may be determined directly as given below.

The residue of the globulin in the test tube in which the precipitation was carried out is washed onto the filter paper by 2 washings with 3 ml each of Na_2SO_4 solution. The globulin in the filter paper is then washed

twice more with 3-ml portions of Na_2SO_4 solution. The funnel is now inserted into a 25- to 30-ml test tube. Puncture a small hole in the bottom of the filter paper and wash down all the globulin with approximately 0.01*N* NaOH. Then unfold the paper and wash off any adhering protein into the test tube. The total volume resulting from this should not be more than 15 or 20 ml. Now add 2 ml of 5*N* NaOH and heat in the boiling water bath for 10 minutes. Cool, and decant into a 50-ml volumetric flask, and wash out with a few ml of water. Add 3 ml of phenol reagent, make up to volume and compare as usual against a standard containing 3 to 5 ml of the standard tyrosine solution.

Fibrin. The fibrin from oxalated blood is separated after the manner of Cullen and Van Slyke. Pipette 1 ml of plasma into a 50-ml cylinder, and add 30 or 40 ml of 0.9 per cent NaCl and 1 ml of 2.5 per cent $CaCl_2$ solution (prepared from the anhydrous salt). Mix the contents and allow to stand for 20 to 30 minutes. The clot is picked up by gently rotating a glass rod through the clotted solution. Place the clot on a dry piece of filter paper and press out the adhering liquor as completely as possible. Introduce the dry protein into a conical 15-ml centrifuge tube, and add 10 ml of water and 1 ml of 5*N* NaOH. Now mix and heat the tube in the boiling water-bath for 10 minutes. The fibrin will be dissolved, leaving behind a suspension of calcium oxalate. This is centrifuged down and the supernatant liquid is transferred to a 25-ml volumetric flask. Wash out the tube with water and transfer the wash water to the volumetric flask through a small filter to prevent the transfer of calcium oxalate. Now add 1.5 ml of phenol reagent and compare against a standard of 3 ml of tyrosine solution contained in a 50-ml volumetric flask.

Conversion Factors. The factors necessary to convert the colorimetric readings to their respective protein values are given in Table 7.

Table 7. Tyrosine Equivalents for Converting Colorimetric Readings to Grams of Protein per 100 ml

		Total Protein	Albumin	Globulin	Fibrin
		Factors of Greenberg and Mirolubova [129]			
Human or Rat	F	11.5	11.8	10.5	11.55
	A	5.75	4.72	2.10	0.347
Dog	F	11.35	11.6	10.05	11.1
	A	5.68	4.64	2.01	0.333
		Factors of Cameron, Guthrie, and White [130]			
Human	F	11.05	11.6	10.2	—
Ox	F	11.1	11.5	10.0	10.7
Sheep	F	11.0	11.2		

The factors under the heading *F* are used in the equation:

$$\frac{S}{U} \times T \times \frac{100}{V} \times \frac{F}{1000} = \text{grams protein per 100 ml}$$

in which S is the setting of the standard, U is the reading of the unknown, T is the mg of tyrosine in the standard solution, V is the aliquot volume of serum or plasma used, and F is the factor for the particular protein fraction being analyzed. If the values of T are maintained at the quantities of 1 mg of tyrosine for the analysis of total protein, albumin, and globulin, and 0.6 mg for fibrin, and the sample values of V are kept at 0.2 ml for total protein, 0.25 ml for albumin, 0.5 ml for globulin, and 1 ml for fibrin, then the equation may be reduced to the form

$$\frac{S}{U} \times A = \text{grams protein per 100 ml}$$

The values of the A factors are given in Table 7.

Remarks. Important points in the determination of the plasma proteins by means of the phenol reagent are the period of heating and the exact composition of the phenol reagent. Heating enhances the chromogenic value of the plasma protein with the phenol reagent. If the older Folin phenol reagent is employed, which is not stabilized by Li_2SO_4, or if the hydroxide concentration is too low, turbid solutions may result that are difficult to read in the colorimeter. Minot and Keller [131] have noticed a slight tendency for the solution to cloud if the room temperature is abnormally high, even with the improved Folin-Ciocalteau reagent.

Apparently the nature of the salt used to salt-out the serum proteins does not affect the color factors of the proteins. Cameron, Guthrie, and White [130] employed $(NH_4)_2SO_4$ for salting out the serum proteins and derived essentially the same factors as those given by Greenberg and Mirolubova (Table 7).

Agreement as to the value of the protein color factors is not universal Employing no radically different techniques, Andersch and Gibson [132] and Minot and Keller [131] derived factors of the order of 1 mg of tyrosine = 13.0 to 13.4 mg of serum protein. The reason for this difference is not clear.

The protein content of cerebrospinal fluid and of the urine may be determined with the phenol reagent in the same manner as described for total protein. In the case of spinal fluid, the factor for total protein should be employed and for urine the albumin factor.

Determination of Fibrin

Fibrinogen may be separated from blood plasma by salting out with saturated NaCl, $\frac{1}{4}$ saturation of $(NH_4)_2SO_4$, 10.6 per cent Na_2SO_4, or 12 per cent Na_2SO_3 solution. Some question has been raised as to whether or not all these salting-out procedures give a quantitative separation of the fibrinogen from the globulin. A convenient method of isolating fibrin is to allow it to clot in recalcified plasma.[121, 133] The clotting procedure of Cullen and Van Slyke has been described in connection with the phenol reagent method for determining plasma proteins (p. 140). The quantity of isolated fibrinogen or fibrin may be determined by gravimetric, micro-Kjeldahl, and colorimetric methods.

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Chapter VII

Relation of Amino Acids and Their Derivatives to Immunity

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Born in Bovenden, Germany, in 1840 and died in 1912. His chief interest and research was in agricultural chemistry. He discovered glutamine, phenylalanine and arginine, and established the constitution of leucine. He had many outstanding pupils and published extensively.

At the outset it may be well to emphasize that the simple amino acids are only indirectly involved in the complex phenomena of immunity, in which their more highly polymerized derivatives, the proteins, together with certain carbohydrates and possibly lipids, are of paramount importance.

Landsteiner, for example, has shown that amino acids may function as haptens, or determinants of a new specificity, characteristic of the amino acid, if these are indirectly coupled to proteins through azo groups.[1] This

function also extends to simple peptides, and in these, as shown by their cross-reactivity, the terminal amino acid exerts a more dominant effect on the specificity than the others in the chain. However, the ability of amino acids to function as haptens when coupled to proteins in this way is by no means characteristic of amino acids, but is shared by many other classes of substances.

While most other studies on the relation of amino-acid derivatives to immunity have been concerned with intact proteins, this has not been true in the related field of allergy, that "altered state" or hypersensitivity responsible for conditions such as urticarias, hay fever, and asthma.[1a] Allergy appears to be a kind of way-station on the road to immunity, at which some subjects stop entirely, while in some animals, such as the guinea pig, the allergic state and immunity may coexist. At all events, some of the substances responsible for the allergic state, such as the active "allergens" of ragweed pollen [2] and of cottonseed [3] and the castor bean [4] appear to be protein derivatives of relatively small size, not exceeding a few thousand in molecular weight. It is uncertain whether or not these correspond to the vaguely defined group of substances formerly called proteoses, or whether they represent characteristic unit combinations of amino acids which may, under other conditions, be polymerized into full-sized proteins.

In view, therefore, of the indirect connection of amino acids with the phenomena of immunity, it would seem best to make a broad, general survey of the chemical basis of these phenomena, leaving it to the reader to fit into this general framework additional pertinent data regarding the amino acids as they appear. For this purpose, the admittedly narrow viewpoint of the chemist permits the oversimplification to be made that the complex phenomena of immunity result from the interplay of antigens and antibodies, the former acting as foreign agents which stimulate in the invaded animal host the formation of the latter.

It was frequently stated in the older immunological literature "horse-serum was used as antigen." But looking now at horse-serum through modern eyes such as the Tiselius electrophoresis apparatus, with its improved optical and photographic recording devices, we see a peak due to a refractive-index maximum at the boundary of each protein component moving in the electric current with a different velocity. There are four peaks, due to albumin and the so-called α, β, and γ-globulins. Like horse-serum, most of the natural sources of antigens are mixtures.

If an antigen, horse-serum albumin, for example, is injected into a rabbit, new substances called antibodies may appear in the animal's serum, and these new, modified serum globulins possess the property of combining chemically, that is, reacting specifically, with the antigen used to stimulate their production. If enough antibody was produced, a precipitate forms as a result of the combination; if the reacting system is very dilute, the mix-

ture of antigen and antibody remains clear; but the combination may be made evident by indirect means, such as the uptake or fixation of complement, an unstable group of serum components which is often taken up by immune combinations (see p. 156). The combination of antigen and the corresponding antibody is specific, for an unrelated albumin such as egg albumin does not react; nor do antibodies to egg albumin, readily produced by injections of this antigen, react with horse-serum albumin.

Like these examples, many antigens are proteins and most proteins are antigens, although the reasons for this activity are not fully understood. In spite of the dominant influence of the specific polysaccharides in many instances of bacterial specificity, particularly among the encapsulated microorganisms, it has not been possible to relate the often appreciable carbohydrate content of proteins to their specificity.[5] Gelatin, which lacks aromatic amino acids, is not antigenic, but this substance is a drastically treated degradation product. Incomplete, but antigenic proteins are casein, which is low in cystine and glycine but contains the aromatic amino acids, and zein, which is low in tryptophane, lysine and glycine, but contains tyrosine and phenylalanine. Even a denatured protein may function as an antigen if it can be brought into solution, and its specificity differs from that of the native protein from which it is derived.[6] An instance is known in which a high molecular weight polypeptide of *d*(-)-glutamic acid [7] is capable of reacting with antibodies stimulated by injection of the anthrax-*mesentericus* group of microorganisms, and actually occurs as part of the capsular material of this group of bacilli.

Detailed studies by Landsteiner and others have shown that substitution in the tyrosine and probably histidine groupings of proteins may change the original specificity into one characteristic of the entering group or radical, particularly if this contains —COOH, —SO_3H, or —AsO_3H substituents. Combination of the free amino groups of proteins with a variety of reagents also alters the specificity, as does the multiple insertion of new groupings at almost any series of reactive centers in the molecule.

It is uncertain whether or not change of shape in itself, such as the uncoiling of a protein during denaturation, may suffice to explain the change in specificity which occurs. Protein structure is such that bundles of polypeptide chains may be held together in a kind of fabric,[8] in part at least by hydrogen bonds,[9] and the surprisingly high percentage of hydroxy-amino acids in many proteins [10] may furnish the requisite groupings for such bonding, with possibly many irregularities, into overall ellipsoid, rod-like, or disc-like shapes. It is probable that various groupings of amino acids, like or unlike, recur at intervals on the reactive surfaces of these aggregations, and that these determine the characteristic chemical, physical, and hence immunological properties of a protein. If such groupings occur more than once, as seems indicated, the protein may be said to be multivalent with respect to a significant chemical or immunologically reactive grouping.

With regard to antibodies, although their nature as modified serum globulins is established, the mechanism and location of their formation in the animal body are uncertain. Parenterally injected antigens, if in particulate form, may be followed under the microscope and are found to be taken up by the cells of the so-called reticulo-endothelial system, in the liver, capillary linings, lymph glands, spleen, and bone marrow, for example. There is little experimental evidence as to what follows, but a number of hypotheses are available. The old one of Buchner, that antibodies are specific owing to actual incorporation of antigen fragments in them, is discredited because like more plausibly repels like than attracts, and because of the inordinately large amounts of antibody formed as a result of stimulation with very small quantities of antigen.

The most plausible theory of antibody formation is that of Breinl and Haurowitz,[11] later presented in more graphic form by Mudd [12] and by Pauling.[13] According to this, antigen or its immunologically reactive fragments penetrate to the site of globulin synthesis, wherever that may be, and by their presence so modify the synthesis of new globulins that this new protein, if it ever happens to come in contact with antigen again, may react with it. In Pauling's version, the configuration of the end groups of the globulin is modified, as the synthesized protein is spun out, by coiling around immunologically reactive groupings on the antigen in a manner characteristic of its surface, so that reaction through surface approximation later becomes possible. This hypothesis, however, entails the difficulty that there is no particular reason why the modified globulin should break loose from the antigen template, and it also requires the presence of antigen at the site of globulin synthesis as long as antibody is formed. Burnet [14] avoided the latter difficulty by assuming that the antigen modifies the intracellular enzyme of globulin synthesis, gaining the further advantage that such a process might continue on reimmunization and cause the progressive changes often observed in antibodies under such conditions. Dr. Florence Sabin [15] has cited evidence that globulins may originate in the surface films given off by macrophages, and that modified globulins or antibodies might be produced by these cells after ingestion of antigen particles, a view compatible with any of the current theories. Experimental evidence in favor of one or the other of these or any alternative theory is badly needed if we are to understand the source and origin of antibodies.

That antibodies are actually modified serum globulins has been amply demonstrated since the introduction of quantitative analytical methods for their measurement [16] and their isolation in appreciable amount [17,18] and study under ultracentrifugation, electrophoresis, and diffusion.[19] It has also been shown by the isotope method that antibody in the actively immunized animal takes up dietary nitrogen in the same way as the serum globulins, with similar distribution of N^{15} among the constituent amino acids. Passively injected antibody, even from the same species of animal,

does not enter into a similar exchange with dietary nitrogen.[20] This finding, with its separation from the same serum of weighable amounts of specific precipitate, one containing heavy N and the other showing none of this isotope, would seem to dispose once and for all of the old bogey that antibody might be merely an unknown substance adsorbed on serum globulin. Large quantities of specific precipitates formed from pneumococcus specific polysaccharides and horse antipneumococcus sera have been washed free from non-specific serum proteins and subjected to analysis for their amino-acid content.[21] The values so found differed little from those observed on hydrolysis of typical horse-serum globulins.

Since antibodies behave as actual proteins, there is the possibility that, like antigens, they carry more than one immunologically reactive grouping on the large molecule. Like the serum globulins, many antibodies have a molecular weight of about 150,000 and some approach 1,000,000 in size. The presence of two or more reactive groupings on the antibody molecule would therefore not seem a far-fetched assumption, making antibody, as well as antigen, multivalent. This assumption provides for the first time a simple and reasonable explanation for specific precipitation [22] and specific bacterial agglutination.[23] In the combination of mutually multivalent antigen and antibody, it will matter little whether one antigen molecule has already combined with one antibody molecule or not, for other reactive groupings on the molecules of each would be available for further reaction. In this way, huge aggregates would be built up, and these would either separate from solution of their own weight or be rendered less soluble by the bringing together of opposite ionic charges which would neutralize each other and so diminish the affinity of the particle for water. If the antigen is on the surface of bacteria, then the cells, with their many molecules of multivalent antigen, combine chemically with the multivalent antibody and so agglutinate into large clumps.

In some immune reactions bacteria or other cells may not agglutinate, but are actually dissolved or lysed. In this reaction not only are antigen and antibody necessary, but in addition, an unstable group of serum components known as *complement*. Complement is also taken up in many instances of specific precipitation and agglutination, but is not necessary for these reactions, as it is in lysis. Two independent investigations have recently removed complement from the realm of a mysterious "colloidal state" of serum proteins. One study [24] has shown complement to be weighable by measurement of the quantity of nitrogen added to certain specific precipitates in the presence of sufficient active complement. This has resulted in the recognition of weight and molecular relationships between hemolysin, complement, and the red cell,[25] reagents in the important Bordet-Wassermann reaction and in the demonstration of other antigen-antibody combinations by the indirect method of complement fixation. This study also resulted in the first plausible explanation for the uptake of

complement by antigen-antibody combinations, making use of the hypothesis that these are due to the mutual multivalence of the reactants.

In the other study of complement [26] some of its components have actually been isolated from fresh guinea-pig serum. So-called "midpiece" or first component, was found to be an euglobulin, while the second and fourth components occurred together as a peculiar muco-euglobulin. The remaining, or third component, was distributed among the various fractions. Both investigations indicated roughly the same amount of complement in guinea-pig serum, about 0.6 to 0.9 per cent of the total protein.

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Chapter VIII

Relation of Amino Acids to Biologically Important Products and the Role of Certain Amino Acids in Detoxication

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Eugen Baumann

Born in Cannstatt, Germany, in 1846 and died in 1896. His finding of iodine in the thyroid gland is considered one of the major discoveries in physiological chemistry. His research covered such subjects as thiourea, cyanamid, sarcosine, etc.

Introduction

The concept that the function of amino acids is to serve primarily as structural material for the body, or to state it more graphically, as building blocks for a static protein molecule is, in the light of recent developments, in need of drastic revision. Neither the protein of active tissues, nor the constituent amino acids can any longer be considered as inert or passive entities, but must be regarded as functioning structure. What appears as fixed is probably but a state of dynamic equilibrium. Many proteins have specific physiological activities, functioning as enzymes, hormones, and in other capacities, but in the last analysis the real chemical activity resides in the amino acids and their position in the particular protein.

The physiological utilization of amino acids is not limited to the formation of proteins. The highly important metabolic agent, glutathione, is a tripeptide composed of glutamic acid, cysteine and glycine. Many essential physiological agents such as creatine, thyroxine, and adrenaline are synthesized from amino acids. Glycine, glutamine and cysteine are employed for conjugation with certain chemical compounds which resist the catabolic processes of the body.

A striking similarity exists between amino acids and vitamins. Both take part in important physiological mechanisms; and the lack of an adequate quantity of either results in a more or less defined pathological state unless the organism can synthesize the deficient compound. As an example, lack of ascorbic acid in the diet causes scurvy in the guinea pig but not in the chick; glycine on the contrary, when absent from the food, brings about retarded growth and other injurious effects in the chick, but no demonstrable disturbance in the guinea pig. Only our accustomed mode of thought prevents us from regarding glycine as a vitamin for the chick and considering all the so-called essential amino acids as categorically like the vitamins. The pathological state produced by lack of methionine would undoubtedly be called an avitaminosis if it were not known that this compound has the structure of an amino acid. These facts are not presented as an academic argument, but merely to emphasize that every amino acid is probably as essential for normal physiological function as are the vitamins.

Somewhat less than half of the known amino acids cannot be synthesized by the mammalian organism. These are considered essential, while the remainder which the body can produce in sufficient quantity for normal growth are called nonessential. The choice of these terms is rather unfortunate, since they carry with them the connotation that the one group is more important or physiologically more significant than the other. Actually it may well be that at least quantitatively some of the non-essential amino acids, such as glycine, play an indispensable rôle in some of the most basic mechanisms, as for example muscle contraction. The very fact that the body possesses a large capacity to produce glycine is perhaps an index of its quantitative importance in metabolism. By way of comparison it may be cited that ascorbic acid is synthesized by the rat and the chick but not by the guinea pig and man. This does not imply that ascorbic acid is less essential (in the sense that it is less dispensable) in the former than in man or the guinea pig. Actually glycine may be more widely used in mammals than in chicks, but its deficiency becomes more easily manifest in the avian organism.

Amino Acids Participating in Conjugation Mechanisms

To understand the significance of amino acids in the conjugation processes, which are generally referred to as detoxication reactions, a brief review is needed of the chemical responses of the body to the presence of

organic compounds that are not normal constituents of food. The four main actions employed by the body on both normal and abnormal compounds that gain entrance are oxidation, reduction, hydrolysis and synthesis (or in a more restricted sense, conjugation). Of these, oxidation is the most important reaction employed by the organism and is the most important means of ridding the body of unessential and harmful substances and of preventing the accumulation of metabolic products. A large number of compounds, including ethyl alcohol and many well-known drugs, are completely burned, and many others are oxidized to compounds which the body can then excrete or combine with a metabolic constituent, as for example glycine with benzoic acid. Reduction is of limited applicability. The conversion of quinic acid, a relatively common acid of plants, to benzoic acid is a good example of this type of reaction. Hydrolysis likewise appears to be of minor importance.

The synthetic or conjugation mechanisms can be considered to be of paramount importance, and it seems that their rôle in physiological functions has been underestimated. The chief substances employed by various species are glycine, cysteine, glutamine, ornithine, glucuronic acid, sulfuric acid, acetic acid and the methyl group. Since there is evidence that the methyl group is derived from methionine, and that even glucuronic acid and sulfuric acid may have as precursors amino acids, it becomes obvious that the conjugation mechanisms depend to a large extent upon the amino acids.

The conjugation reactions are generally regarded as synonymous with detoxication mechanisms. Sherwin [1] expresses most concisely the prevailing view that dominated biochemical thought for many decades. He states: "It has been necessary for the body to call to its aid a chemical defense mechanism to guard against poisons absorbed from the gastro-intestinal tract. After many generations this chemical defense mechanism has been so perfected in its battle against putrefaction products absorbed from the intestine that it is now quite able to cope with many of the foreign organic compounds." The writer since 1927 has opposed the view that the conjugation reactions are designed primarily for detoxication. He believes that conjugations are normal metabolic processes made manifest because the body is applying them to a foreign compound which at some stage resists ultimate catabolism, becoming so to speak a physiological clinker and leaving a recognizable end-product. Detoxication is incidental, and perhaps even accidental. In some instances the conjugation may actually increase the toxicity of the substance, as illustrated by the acetylates of various sulfonamide compounds.[2] Sammons and Williams [3] have recently expressed a similar view: "They (conjugation reactions) need not be special mechanisms at all, but mechanisms which are used normally by the body and which are revealed in exaggerated or possibly modified form when foreign organic compounds are dealt with."

The reasons why conjugation mechanisms cannot be considered primarily as detoxication processes are: (1) The conjugated end-product is often no less toxic than the original substance. There is no evidence, for example, that benzoic acid is more noxious than hippuric acid. As already mentioned, some acetylated sulfonamides are more toxic than the sulfonamides themselves. (2) Foreign compounds purely synthetic in origin that have never existed in nature and with which the organism in its development never could have come in contact are conjugated as readily as substances constantly formed in intestinal putrefaction. It is inconceivable that the organism could have anticipated the laboratory production of chloral and perfected a specialized enzymic machinery for its reduction to trichloroethyl alcohol and subsequent conjugation. (3) Essential food elements such as *p*-aminobenzoic acid and nicotinic acid, both of which are now recognized as vitamins, undergo conjugation, which can hardly be for the purpose of detoxication.

These remarks should not gainsay the fact that a detoxication of many compounds is effected by conjugation. This may be accomplished by the neutralization of the active group responsible for the physiological activity of the compound by coupling it with glycine or one of the other compounds available for this purpose. Significantly, many of the conjugated products are much stronger acids than the mother substance, and usually strong acids are excreted with greater ease than weak ones; examples are benzoic acid and hippuric acid, phenol and phenol sulfuric acid, menthol and menthol-glucuronic acid. It is becoming more evident that the conjugation mechanisms are utilized in the control of hormonal activity, especially of the sex hormones, and in this capacity the conjugation process appears to play a true detoxifying rôle.

The presentation of the amino acids utilized for conjugation will be from the point of view that the reactions which the amino acids manifest through a foreign organic compound have their counterpart in normal physiological mechanisms. A discussion of metabolism and the known physiological actions of the several amino acids will precede the treatment of their conjugation.

Glycine Metabolism. In the mammalian organism glycine is readily synthesized. On the basis of the amount produced for conjugation with benzoic acid, it has been found [4] that man can mobilize 9 mg per hour per kilo of body weight, the pig 15 mg and the rabbit 25 mg. It is probable that the maximum capacity is much higher than these figures indicate. The actual chemistry of the synthesis of glycine is still unsolved. Swanson,[5] confirming the finding of Lewis and others, showed that the glycine produced for conjugation with benzoic acid can be synthesized from nitrogen which would otherwise be excreted as urea. The source of the two-carbon chain is still uncertain. Glyoxalic acid (CHOCOOH) and glycolic acid ($CH_2OHCOOH$) have been postulated, but no satisfactory experimental

proof has been furnished. Since acetic acid serves as a substitute for glycine in the diet of the chick,[6] this acid deserves further study. The simple structure of glycine makes it appear probable that the precursor must likewise be a fairly elementary compound.

The problem of outlining the catabolism of glycine is likewise a tantalizing task. While it appears exceedingly simple, it is in reality complex and confusing. The general assumption is that glycine is readily deaminated; but Bach,[7] studying the reaction with tissue slices of organs, found that glycine, unlike alanine, is not deaminated. He postulates that "glycine condenses with various groups such as α-keto acids acting as a fixative for keto compounds, producing an equilibrium effect in systems responsible for the formation of such compounds." Ratner and his associates [8] have recently described a glycine oxidase, a flavo-protein which brings about oxidative deamination:

$$CH_2NH_2COOH + \tfrac{1}{2}\,O_2 \longrightarrow CHOCOOH + NH_3$$

This enzyme is found in the kidneys of various animals and also in the liver of the rabbit.

Since the well-known work of Ringer and Lusk in which a quantitative formation of glucose from glycine was found in phlorizinized dogs, it has been unhesitatingly accepted that this amino acid belongs to the sugar-forming group. This is challenged by the work of Reid,[9] who found that whereas alanine increases unequivocally the store of glycogen, injected glycine under the same conditions does not. Olsen, Hemingway and Nier,[10] using glycine containing the stable isotope of carbon, found that some of the isotope appeared in the liver glycogen, but the increase was much greater than could be accounted for on the basis of glycine alone. Such results indicate the difficulty of interpreting these types of metabolic data. The paucity of concrete information concerning the metabolism of glycine is a serious obstacle in arriving at a better understanding of its conjugation reaction. As a matter of fact, it may well be that the latter may ultimately become the means of solving the problem of the intermediary metabolism of glycine.

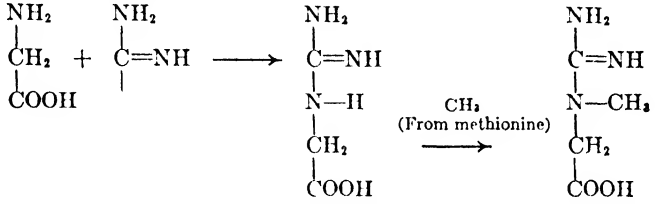
Glycine has a high specific dynamic action, which means, according to Kriss [11] and others, that much heat is evolved in its intermediary metabolism, and not, as some have held, that it produces a metabolic stimulation in a pharmacological sense.

Function of glycine. In the body this amino acid has at least four distinct functions: (1) it is a constituent of many proteins; (2) it combines with cholic acid to form the bile acid, glycocholic acid; (3) it takes an integral part in the synthesis of creatine; and (4) it conjugates with a large number of aromatic acids.

It is interesting that glycine is particularly abundant in such inert proteins as collagen (gelatin), elastin, silk fibroin and chicken feathers. There is no evidence that the glycine in proteins is directly or readily available for the other metabolic functions listed.

The conjugation of glycine with cholic acid is interesting since the carboxyl group is terminal to a long aliphatic chain and not adjacent to an aromatic nucleus as in benzoic acid. Whether the mechanism employed for conjugation of cholic acid with glycine is the same as that for the production of hippuric acid is not known, but it is significant that the liver of the dog lacks the ability to synthesize hippuric acid, and that the bile of this animal contains little or no glycocholic acid. Oddly enough when glycine is fed with egg albumin it nevertheless appears to stimulate bile acid production.[12]

Of fundamental importance is the rôle played by glycine in the synthesis of creatine. The reaction established by the use of isotope nitrogen [13] and by tissue-slice technique [14] is:



Glycine (*From arginine*) *Guanidino-acetic acid* *Creatine*

Since creatine (as creatine phosphate) is essential for muscle contraction, the ability of the organism to synthesize glycine is of fundamental importance. In mammals it proceeds with such efficiency that only under extremely adverse conditions can it be disturbed. Rats given benzoic acid daily in their diet fail to grow.[15] In the light of later work on chicks given a diet low in glycine, it appears likely that benzoic acid deprives the rat of glycine, thus causing interference with the synthesis of creatine. The chick is unable to synthesize sufficient glycine for normal growth,[6] and must therefore depend on obtaining it from the diet. When given a glycine-poor ration, the chick exhibits poor growth, profound weakness simulating paralysis, and has a significantly low concentration of muscle creatine. Feeding creatine prevents the syndrome.

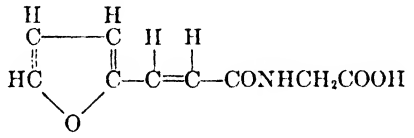
The chick also lacks the ability to synthesize arginine; therefore the production of creatine is likewise limited by this amino acid. This topic will be more fully discussed under ornithine.

In view of the importance of glycine in the synthesis of creatine, the possible therapeutic value of this amino acid in muscle diseases, particularly progressive muscular dystrophy and myasthenia gravis, has received considerable study. The early optimistic results have not been substantiated by later investigators and the status of glycine therapy for this group of diseases is uncertain today.[16]

Conjugation. Glycine is combined with benzoic acid to form hippuric acid by nearly all species of vertebrates studied except birds and reptiles, which use ornithine. Even in the lower forms, such as frogs and turtles, the hippuric acid synthesis occurs, showing that it is indeed a fundamental reac-

tion. The synthesis is brought about enzymatically in a manner perhaps similar to that employed by the body to unite amino acids to form peptides. Each contains the CONH group, and the free energy of formation of hippuric acid is approximately the same as of the peptide bond, as Borsook and Dubnoff [17] have recently found. These authors point out that the synthesis of hippuric acid *in vivo* must therefore be a coupled reaction, of which one of the components must be energy-donating.

The conjugation of glycine with foreign organic compounds is limited to cyclic structures containing a carboxyl group. To the writer's knowledge no aliphatic acid has been found to conjugate with glycine in the body. The carboxyl group which conjugates with glycine usually is directly attached to the aromatic ring as in benzoic acid, or separated by a CH_2 group (phenylacetic acid). The notable exception is cholic acid, in which the carboxy group is at the end of a 4-carbon chain. When phenyl-substituted aliphatic acids are fed, they are reduced by β oxidation to phenylacetic or benzoic acid. There is some evidence, however, that cinnamic acid when fed is not all oxidized to benzoic acid but that a fraction is conjugated with glycine. Sasaki [18] reported the interesting observation that furfurylpropionic acid, when fed to a dog, is excreted as furfurylacryluric acid:

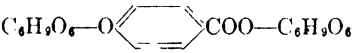


Although the statement is made that glycine conjugates with over a hundred different acids, a survey of the literature would show that perhaps less than thirty have been isolated and studied. Only a few types need be mentioned to illustrate the known factor that influences the conjugation with glycine.

The first important structural influence is the distance of the carboxyl group from the aromatic ring. This is brought out by the marked difference with which benzoic acid and phenylacetic acid are handled in the organism. In the dog, the liver lacks the ability to conjugate benzoic acid with glycine, whereas the organ can synthesize phenaceturic acid.[19] Thus two separate and distinct mechanisms exist for the conjugation of these two acids in the dog. In the human this is even more striking, since phenylacetic acid is combined with glutamine. The fact that the synthesis of phenylaceturic acid in the dog occurs in the liver whereas the production of hippuric acid occurs exclusively in the kidneys helps to explain why less hippuric acid is synthesized and why extra glycine in the diet increases phenaceturic acid more than hippuric acid. Only the glycine which reaches the kidney can influence the output of hippuric acid, while the production of phenylaceturic acid is benefited by the fact that glycine is very probably synthesized in the liver and that ingested glycine reaches this organ first.

Both benzoic acid and phenylacetic acid are partly conjugated with glucuronic acid. In the dog roughly 70 per cent of the benzoic acid is combined with the latter and only 30 per cent is excreted as hippuric acid. There is evidence that glucuronic acid is produced in the liver; consequently a large proportion of the benzoic acid is converted to benzoyl-glucuronic acid before it leaves the liver and reaches the kidney. The physiological purpose of this dual conjugation is not known but presents an interesting problem that may ultimately shed much light on intermediary metabolism. In man very little glucuronic acid is conjugated with either benzoic or phenylacetic acid.

The second important structural influence is the type of the aromatic nucleus and the groups it contains and the position they occupy. Most of our knowledge concerns substituted benzoic acids; but even in this limited field the information is too fragmentary to attempt any extensive correlation. A few striking findings [20] can be pointed out. *p*-Hydroxybenzoic acid in the dog is conjugated to a small extent with glycine and the remainder with glucuronic acid, but curiously the hydroxy group is also combined with glucuronic acid forming the unusual compound, glucuronide of *p*-hydroxybenzoyl glucuronide:

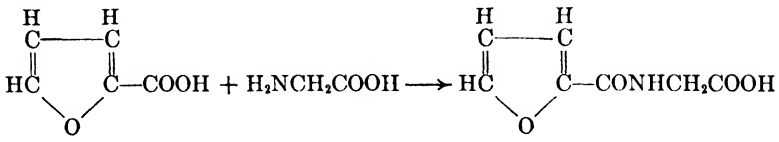


It is unusual to find two groups of a compound becoming conjugated. In man *p*-hydroxybenzoic acid is excreted partly unchanged, and the remainder combined with glycine, whereas *p*-methoxybenzoic acid is conjugated to the same extent as the *p*-hydroxy acid with glycine, but the remaining portion is combined with glucuronic acid. The aminobenzoic acids in the dog are combined principally with glucuronic acid, but in man and the rabbit they are mainly acetylated.

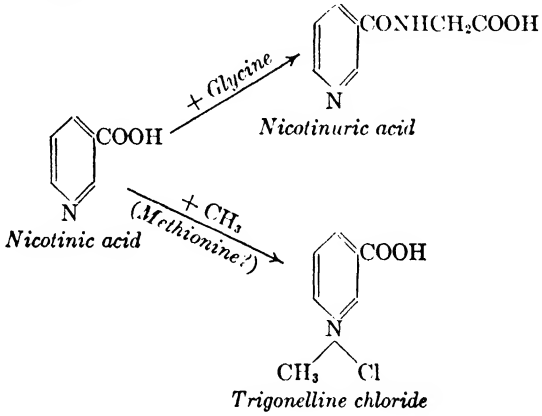
Substitution in the ortho position exerts a marked inhibitory effect on the conjugation of benzoic acid with glycine, irrespective of the nature of the substituting group. Ellis and Walker [21] have recently found that the hydrolysis by hippuricase is markedly inhibited by ortho-substitution of the hippuric acid. They offer evidence that the ortho group does not prevent combination with the enzyme, but inhibits breaking of the peptide linkage. In the conjugation with glycine the ortho-substituted benzoic acid can likewise combine with the enzyme and temporarily inactivate it, as illustrated by the fact that production of *p*-methoxyhippuric acid is depressed if *p*- and *o*-methylbenzoic acids are fed together.[20] This peculiar inhibitory action of ortho-substitution perhaps explains the marked pharmacological and toxic action of various drugs. Salicylic acid is perhaps the best known example of an ortho-substituted benzoic acid.

Information is meager concerning the influence of aromatic nuclei other than benzene. β-Naphthoic acid is readily converted to β-naphthuric acid, but α-naphthoic resists conjugation, since the second benzene ring has the

same effect as substitution in the ortho position. Pyromucic acid is coupled with glycine:

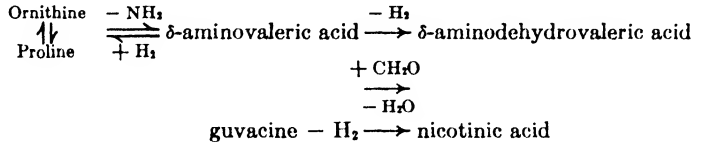


The conjugation of nicotinic acid deserves special consideration. In man, dog, rat, and undoubtedly in many other animals, the acid in part undergoes conjugation with glycine and in part is methylated, presumably with methionine as the agent:



Whether these reactions are solely for the purpose of eliminating nicotinic acid, or represent initial steps for its metabolic utilization is not known. The latter possibly might profitably be further investigated, even though it has been found that neither trigonelline nor nicotinuric acid is curative for dogs suffering from blacktongue.

Various species possess the ability to synthesize nicotinic acid, and the following scheme has been postulated by Guggenheim [22] to explain its formation.



There is thus a possibility that the metabolism of nicotinic acid is associated with four amino acids: glycine, methionine, ornithine and proline. The close relationship of amino acids to this particular vitamin suggests that it is not improbable that further scrutiny may disclose other relations between these two groups of compounds. The biological synthesis of *p*-aminobenzoic acid presents the problem of how the amino group is intro-

duced into the benzene ring. Could tyrosine be the precursor of this vitamin?

Uric acid, both in its synthesis and in its excretion, is intimately linked with the amino acids. Both benzoic acid and phenylacetic acid depress the excretion of this catabolic end product, and their effect is promptly abolished when they are conjugated and excreted. Excretion of uric acid is also depressed by lactic acid, glycolic acid, acetoacetic acid and by ketosis, whereas it is stimulated by glycine, alanine, aspartic acid, glutamic acid and pyruvic acid.[23] The effect of benzoic acid and phenylacetic acid is specific on uric acid, since the excretion of other nitrogenous products is not inhibited. Salicylic acid, and to a lesser degree, *p*-hydroxybenzoic acid, unlike benzoic acid and most other substituted benzoic acids, stimulates the excretion of uric acid. Since uric acid still remains one of the most enigmatic products of metabolism, a correlation of these findings is a difficult task; and yet without an answer to these problems, no rational approach to a better understanding of such clinical conditions as gout appears likely.

The conjugation of benzoic acid with glycine has become a widely used test of liver function since its introduction in 1933.[24] When sodium benzoate is administered to a human subject, about 1 to 1.5 gm of hippuric acid (equivalent roughly to 0.7 to 1.0 gm of benzoic acid) are excreted per hour. The output is remarkably constant, because the organism is producing the required glycine at a maximum constant rate. The synthesis of the latter occurs mainly or perhaps exclusively in the liver; therefore, when the organ is injured and its function impaired, the output of hippuric acid is decreased. The production of hippuric acid has been found by numerous clinical investigations to be a fairly reliable quantitative measure of liver damage. A significant diminution occurs in catarrhal jaundice and other types of hepatitis, in atrophic cirrhosis, in long-standing obstructive jaundice, in malignancy of the liver and other less well defined pathological conditions of the organ. The test is particularly satisfactory for prognosis and to follow the effects of treatment. The most serious interfering factor is impairment of kidney function, but fortunately the excretory capacity of the kidney for hippuric acid is much greater than the synthetic power of the liver, so that only severe kidney disease will invalidate the test. In liver injury both the synthesis of glycine and the conjugating mechanism are usually impaired, so that even though glycine is administered the production of hippuric acid is not always restored to normal. In normal individuals part of the glycine is derived from exogenous sources, but most of it is synthesized even when an excess is supplied. This has been convincingly demonstrated by Rittenberg and Schoenheimer[25] using isotopic nitrogen.

Benzoic acid has no marked toxic action even in patients whose capacity to synthesize hippuric acid is greatly diminished. It is improbable there-

fore that the conjugation is primarily one of detoxication. Hippuric acid, however, is readily excreted, whereas benzoic acid for reasons not yet known does not appear free in human urine even after the ingestion of large amounts of benzoic acid.

It might be mentioned that other detoxication reactions such as glucuronic acid production, have been proposed from time to time to test liver function but none has won wide acceptance. It is nevertheless wise not to ignore the possibility that tests as good as or better than the hippuric acid synthesis may be developed on the basis of these conjugation mechanisms.

Ornithine ($NH_2(CH_2)_3CHNH_2COOH$). In 1877 Jaffé discovered that benzoic acid is conjugated in the hen with ornithine, an α-amino acid which has not been found in proteins, but which is closely related to naturally occurring arginine. For many years the ornithine conjugation was regarded as a unique or odd type of reaction unrelated to any known normal physiological mechanism, since this amino acid was not encountered in the mammalian organism.

Ornithine has assumed a new place of importance since the work of Krebs and Henseleit in 1932;[26] on the basis of results obtained from tissue-slice experiments they proposed a scheme to explain the formation of urea (see page 169). Urea, the principal end-product of nitrogen metabolism, is derived from arginine which is acted upon by the enzyme arginase. The resulting ornithine condenses with ammonia and carbon dioxide to form citrulline, which reacts further with a second mole of ammonia to restore the arginine, thus completing the cycle. In the chick, the Krebs-Henseleit cycle is incomplete, as shown by the broken lines in the circle of diagram 1. In this species the ability to convert ornithine to citrulline is lost;[6] therefore arginine cannot be synthesized and so becomes for the avian organism an essential amino acid. Curiously, the fowl can convert citrulline to arginine. One can surmise that since the avian organism does not synthesize urea, its arginine requirements are much less than those of the mammal and can be met by a normal natural diet. In the fowl the end-product of nitrogen metabolism is uric acid, and there is good evidence that ornithine is an intermediary in the formation of this catabolite. In the first place when benzoic acid is fed to chicks, the excretion of uric acid is definitely decreased; presumably the nitrogen which would go into uric acid is excreted as dibenzoylornithine.[27] In the second place, it was observed by Takahashi[28] that when benzoic acid was injected into incubating eggs, no ornithine was formed until the ninth day which, significantly, is about the time when uric acid appears in the embryo.

The facts that uric acid is the end product of nitrogen metabolism in birds and reptiles, and that benzoic acid is conjugated with ornithine present an exceedingly interesting problem in the evolution of metabolic processes. In such lower forms as the frog and turtle, urea and hippuric acid are synthesized and excreted, and the same mechanisms are retained by

the higher mammals and man. Only in birds and reptiles is there this marked metabolic deviation. It has been postulated that in these species the necessity of conserving water has brought about the excretion of nitrogen in the form of the comparatively insoluble uric acid instead of the soluble urea which in its excretion carries with it a relatively large amount of water. Apparently, therefore, the fundamental reaction of the synthesis of urea is abolished by breaking the Krebs-Henseleit cycle between the

The Krebs-Henseleit Cycle*

$$NH_3 + CO_2 + H_2N(CH_2)_3CHNH_2COOH \longrightarrow H_2NCONH(CH_2)_3CHNH_2COOH + H_2O$$

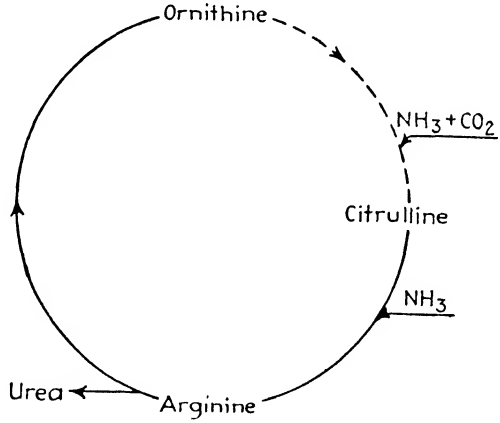
Ornithine — *Citrulline*

$$+ NH_3$$

$$H_2NCONH_2 \longleftarrow H_2NCNH(CH_2)_3CHNH_2COOH + H_2O$$

Urea — *Arginine*

ornithine-citrulline step; and in this manner the power to synthesize arginine is also lost. Perhaps even the inability to form glycine is contingent upon this metabolic gap.



Interestingly, the chick embryo in its early stages can form urea and also glycine as attested by the observation that the egg contains relatively little of this amino acid, whereas the fully developed chick contains over 2 per cent glycine. This makes it appear that the embryo possesses synthetic powers which in a later stage are lost.

The chick combines benzoic acid, phenylacetic acid, pyromucic acid, and probably many other organic acids with ornithine.

$$NH_2(CH_2)_3CHNH_2COOH + 2\ C_6H_5COOH = C_6H_5CONH(CH_2)_3CHNH(COC_6H_5)COOH$$

Ornithine — *Benzoic acid* — *Dibenzoylornithine* or *Ornithuric acid*

* The Krebs-Henseleit cycle must still be regarded as a theory. Although much evidence is in its favor, there are findings which are not in agreement with this concept as Borsook and Dubnoff [30] point out.

Cysteine ($HS—CH_2CHNH_2COOH$). This compound was considered an essential amino acid until Jackson and Block [29] in 1932 showed that methionine can replace it in the diet. It has been established that the body can synthesize it provided the —SH group is supplied. The probable production is as follows:

$$\underset{\textit{Serine}}{HOCH_2CHNH_2COOH} + SH \text{ (from homocysteine)} = \underset{\textit{Cysteine}}{HS—CH_2CHNH_2COOH}$$

Recently evidence has been obtained that methionine cannot directly transfer the sulfhydryl group to serine, but only after its demethylation and conversion to homocysteine can it participate in the synthesis of cysteine.[30]

Cystine and cysteine play an important rôle in metabolism. They occur in such physiologically active proteins as insulin and thrombin, and there is evidence that the activity of these proteins is dependent upon the sulfhydryl group. Paradoxically, cystine is also abundantly present in the most inert proteins of the animal — hair, wool and feathers.

Glutathione is a tripeptide of glutamic acid, cysteine and glycine. Its exact function is still unknown. In association with ascorbic acid and other compounds it takes part in the oxidation and reduction mechanisms of the body. From recent studies in which isotopic nitrogen was employed, it has been learned that the metabolic turnover of glutathione is very rapid [31] and that it appears to participate in the transfer of amino acids into the protein molecule. Various investigators have speculated concerning the rôle of glutathione in the detoxication mechanism since it contains three amino acids used for conjugation, but no supporting evidence has been found. Waelsch and Rittenberg [32] employing isotopic nitrogen concluded from their results that glutathione does not participate directly in the synthesis of hippuric acid.

Taurine is a product of cysteine. Its formation is believed to be as follows:

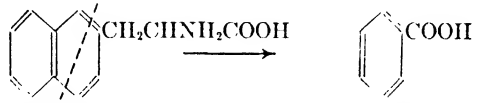
$$\underset{\textit{Cysteine}}{CH_2SH\ CHNH_2\ COOH} \xrightarrow{O_2} \underset{\textit{Cysteic acid}}{CH_2SO_3H\ CHNH_2\ COOH} \longrightarrow \underset{\textit{Taurine}}{CH_2SO_3H\ CH_2NH_2} + CO_2$$

The only known function of taurine is as a component of taurocholic acid. In most species this bile acid and glycocholic acid are present in varying proportions in bile, but in the dog the former appears exclusively.

Cysteine plays an important function in detoxication; but in order to present this subject properly, it seems essential to consider briefly the fate of aromatic hydrocarbons in the body. These compounds are of particular interest from a medical point of view. The simplest representative, namely, benzene, is a well-recognized industrial hazard. It damages the bone mar-

row and causes an aplastic anemia, leucopenia and thrombocytopenic purpura. Naphthalene fed to rabbits causes cataract similar to the senile type occurring in man. Other hydrocarbons of a more complex type possess carcinogenic properties.

The characteristic feature of the unsubstituted benzene ring is its resistance to chemical change *in vitro*. The same refractiveness is exhibited when the compound is exposed to the enzymatic and oxidation mechanisms of the body. The introduction of chemical groups into the ring immediately changes the stability of the nucleus. By attaching the group $-CH_2CH(NH_2)COOH$ or $-CH_2CHOCOOH$, the aromatic ring is catabolized with the same ease as a sugar molecule or the aliphatic chain of a fatty acid. This holds not only for the benzene ring, but also for β-naphthylalanine, in which case ring one is completely metabolized (Kikkoji [33]).



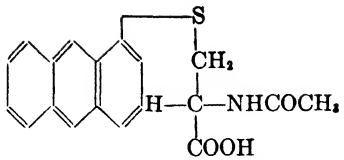
β-Naphthylalanine *Benzoic acid*

Aliphatic groups in the benzene ring are generally oxidized easily. Thus toluene is converted to benzoic acid and therefore differs from benzene in its toxicological action.

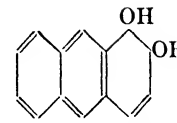
No satisfactory quantitative studies can be found on the fate of benzene in the body, but it has been known since the work of Schultzen and Naunyn in 1867 that phenol is one of the products. Polyphenols such as catechol and quinol are also found, and Jaffé isolated muconic acid (HOOC—CH=CH—CH=CH—COOH), which indicates that the ring can be broken. In benzene poisoning, the excretion of ethereal sulfates increases, and this has been suggested as a means of determining exposure to the chemical.[34]

Baumann and Preusse in 1879 found that when bromobenzene was fed to dogs, it was excreted as *p*-bromophenyl-mercapturic acid [$BrC_6H_4S—CH_2CH(NHCOCH_3)COOH$]. This indicated that the organism attacks the benzene ring by means of the sulfhydryl group. Cysteine becomes directly linked to the aromatic nucleus through the sulfur atom. Subsequently the cysteine becomes acetylated, thus forming the complex called mercapturic acid. Other halogenated hydrocarbons likewise are converted to mercapturic acids, and the same occurs to benzene, naphthalene and anthracene. Only very recently, however, has phenylmercapturic acid been isolated after the administration of benzene.[35]

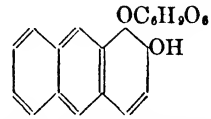
The fate of anthracene is particularly interesting because it offers some suggestions concerning the possible course of events taking place in the degradation of hydrocarbons. Boyland and Levi [36] isolated three derivatives from the urine of dogs fed anthracene:



I. *1-Anthrylmercapturic acid*



II. *1,2-Dihydroxy-1,2-dihydro-anthracene*

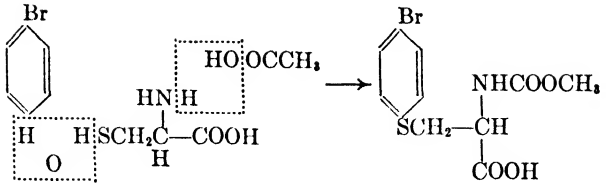


III. *1,2-Dihydroxy-1,2-dihydro-anthracene glucuronide*

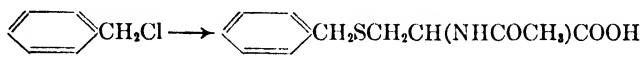
It seems logical to assume that the mercapturic acid (I) is the first product formed and that the introduction of the hydroxy groups (II) is a later step. With the presence of hydroxy radicals, conjugation with sulfuric acid or glucuronic acid (III) is a logical sequence. The writer is inclined to believe that the only means available for the organism to attack the unsubstituted aromatic ring is by means of the sulfhydryl radical. Then the mercapturic acid group is split off by hydrolysis, leaving in its place a hydroxy radical. With the introduction of several hydroxy radicals, the lability of the aromatic nucleus is increased so that ultimately it undergoes complete catabolism.

According to Stekol [37] and others, the organism can utilize cysteine directly for the synthesis of mercapturic acids. Likewise *l*-cystine and *dl*-methionine increase the production of this conjugated product, while taurine does not. When a substance like bromobenzene is fed, part of the cysteine is supplied by the diet; the remainder must come from endogenous sources. If, therefore, bromobenzene is fed continuously a deficiency in cysteine occurs, just as daily administration of benzoic acid leads to a glycine deficit. In both instances a retardation of growth manifests itself. By adding extra amounts of cysteine, cystine or methionine to the diet, the effect of bromobenzene can be counteracted. Glutathione and *dl*-homocysteine likewise promote growth in cysteine deficiency caused by the hydrocarbon, but the effect is not due to a direct detoxication of the drug but to a replenishment of tissue cysteine. It appears that both the hydrocarbon and glutathione compete for the dietary cysteine; but in this contest the mercapturic acid synthesis wins the lion's share, so that ultimately a systemic decrease of glutathione occurs. The lack of this substance in the crystalline lens may lead to the degeneration recognized as cataract, which as has been mentioned, is observed after feeding naphthalene continuously.

The chemistry of the formation of the mercapturic acids is relatively simple:



By oxidation the sulfur becomes attached to the ring and subsequently the amino group of the cysteine radical is acetylated. Stekol [37] has found an example in which the sulfur is attached to an aliphatic group; namely, the mercapturic acid formed from benzyl chloride:



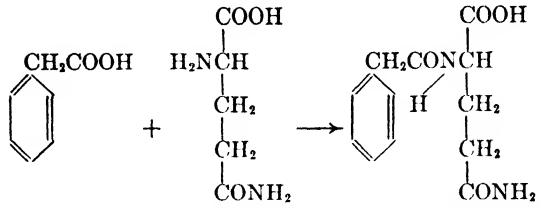
In this case the chlorine is replaced by S and hydrochloric acid is formed.

One can justifiably question whether the synthesis of mercapturic acids is a true detoxication reaction. If it represents the first step in the body's attack on the aromatic nucleus and if the phenolic derivatives result from this reaction, it is very likely that the products are more harmful than the mother substance itself. Halogenated hydrocarbons such as bromobenzene appear to be fairly harmless; their most serious effect seems to be that they deflect cysteine from its normal metabolic path to the mercapturic acid conjugation, thereby robbing the body of an adequate supply of sulfur amino acids. A curious example in which a substance which is supposedly detoxified actually functions as the detoxifying agent is afforded by the use of bromobenzene in selenium intoxication. In this condition selenium replaces sulfur in the cysteine molecule as well as in the other sulfur amino acids. By feeding bromobenzene, this selenium cysteine is conjugated with it and thereby eliminated from the body.[37]

Ethereal sulfate conjugation is not directly dependent upon amino acids. The body appears to be able to conjugate the inorganic sulfate radical with the phenol group, but the sulfuric acid is, to be sure, the end product of the catabolism of the sulfur-amino acids. There is no evidence, however, that either cysteine or methionine is directly concerned with the sulfate conjugation. The hypothesis that the ethereal sulfate is the product of the oxidation of a mercapturic acid is contrary to the expected chemical reaction, for such an acid should yield a sulfonic acid — not a phenolic group esterified with sulfuric acid.

It is interesting that the sulfate conjugation of phenols is influenced by other groups in the ring, especially when in the ortho position.[38] A strong positive group such as —COOH or —NO_2 depresses, a basic group increases the conjugation. The coupling of ortho-substituted benzoic acids with glucuronic acid responds in a similar way, whereas all groups irrespective of their polarity inhibit the conjugation with glycine. Only the latter therefore is a true steric hindrance effect.

Glutamine ($HOOCCHNH_2CH_2CH_2CONH_2$). The most limited conjugation process is that of phenylacetic acid with glutamine, which was discovered by Thierfelder and Sherwin in 1914.



Phenylacetic acid *Glutamine* *Phenylacetylglutamine*

This reaction has been found to occur only in man and the chimpanzee and with no other compound except phenylacetic acid. Even the substituted phenylacetic acids are conjugated by a different mechanism.

Glutamine appears to be synthesized from waste nitrogen, which would otherwise be excreted as urea.[27] The writer[23] has obtained indirect evidence that pyruvic acid and alanine increase the synthesis of glutamine, but that glutamic acid does not. The failure of the latter to accelerate the synthesis of phenylacetylglutamine indicates that the amidination of glutamic acid occurs before the conjugation takes place. Sherwin has found that when phenylacetylglutamic acid is fed, it is not converted by the body to the glutamine derivative.

The fact that the conjugation with glutamine is limited does not necessarily mean that glutamine is of little physiological significance. Örström *et al.*[39] have found that glutamine is readily synthesized by the avian liver from ammonium pyruvate, and they believe that this compound may be an important factor in cell metabolism. These investigators suggest that the carbon chain of glutamine enters directly into the purine ring. It may therefore be a part of the mechanism employed by the bird for the excretion of waste nitrogen as uric acid. Leuthardt[40] has observed that glutamine in guinea-pig liver forms urea, and Bach[41] postulates that this compound plays a part in the mechanism employed by the organism for the production of urea, or in other words, complements the Krebs-Henseleit cycle. The recent report of Harris[42] that blood and spinal fluid normally contain a glutamine-like substance adds further evidence that this compound cannot be ignored in metabolic studies and that its conjugation with phenylacetic acid should not be regarded as a mere physiological curiosity.

Glucuronic acid. This compound can be produced both from the carbohydrate stores of the body and from glycogenic amino acids. When a drug like sodium benzoate is fed to a completely diabetic dog, the organism will utilize the potential glucose derived from glycogenic amino acids for the production of glucuronic acid. On the basis of this finding the writer postulated that glucuronic acid is synthesized from a simple 3-carbon chain compound which is derived either from carbohydrate stores or from amino

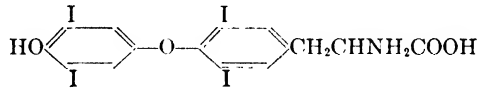
acids such as alanine. In confirmation of this, Lipschitz and Bueding [43] have shown by means of liver slices that the production of glucuronic acid is increased by dihydroxyacetone, pyruvic acid, lactic acid and by insulin.

Perhaps the body uses one simple compound, a triose, for the synthesis of most of the compounds used in conjugation. It can be postulated that by means of one mechanism, this primary triose is converted to glucuronic acid, by another to glutamine, by a third to ornithine and by a fourth to cysteine provided the —SH group is available. Whether glycine is derived from a 3-carbon compound is difficult to decide on a purely chemical basis.

The reactions usually designated as detoxication processes are but a part of the extensive synthetic mechanisms of the body. It is logical to suppose that as source material, the organism employs simple compounds such as carbon dioxide, ammonia, urea, pyruvic acid, and lactic acid. In these syntheses transamination, transmethylation, transamidination, transsulfhydrylation, and shifting of other groups readily occurs.

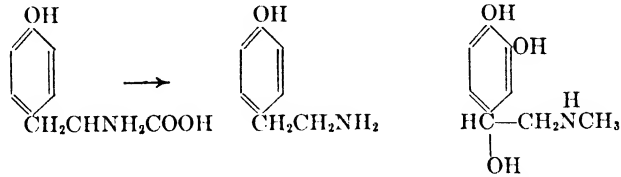
Amino Acids as Precursors of Hormones

Thyroxine. Two molecules of tyrosine are required for the synthesis of thyroxine, which has the formula:



Until recently it was believed that the synthesis of this compound was a specialized function of the thyroid gland, but recently it has been found that by iodination of casein, monoiodotyrosine, diiodotyrosine and thyroxine can be obtained in the hydrolyzate. It has been further observed that diiodotyrosine, when dissolved in dilute sodium hydroxide and the solution adjusted to pH 8.8, is slowly converted to thyroxine.[44]

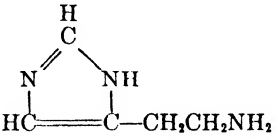
Epinephrine (adrenaline). The mother substance of this hormone is again tyrosine. The first step appears to be decarboxylation, and the resulting tyramine is converted in the adrenal gland by oxidation and methylation to epinephrine.[45]



Tyrosine *Tyramine* *Epinephrine (adrenaline)*

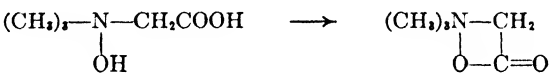
Amines and Betaines. Every amino acid can be decarboxylated and thus made to yield a primary amine. As a result of bacterial action in the intestines, many of these amines are formed, and it was once believed that they were mainly responsible for a clinical condition known as auto-intoxication.

It is probable that the importance of these compounds as exogenous toxins was overestimated. Of the amines derived from amino acids, only two have so far been found to play a rôle in normal physiology. They are tyramine and histamine. The former is an intermediary in the synthesis of epinephrine; the latter can be classed as a hormone. Histamine has the structure:



The amount of histamine absorbed from the gastrointestinal tract probably is small compared to the amount which is produced in the body. It is employed as the hormone of gastric secretion, and it appears suddenly in large amounts during anaphylactic shock. It is generally accepted that the precursor of histamine is *l*-histidine. Injection of the latter substance increases the histamine content of the body. An extensive literature on this subject has developed.[46, 47]

Betaine. This compound is an inner ammonium salt derived from trimethylglycine:



It occurs in the sap of the sugar beet (*Beta vulgaris*); hence its name. When ingested by animals, it is not utilized, but shows no poisonous qualities. Structurally the compound is closely related to choline ($(CH_3)_3NOHCH_2CH_2OH$), but the writer failed to find any references in the literature suggesting that glycine might be a precursor of choline.

The betaines of other amino acids have been found in plants, but too little is known of their behavior in the mammalian organism to warrant discussion of this topic.

Summary

The amino acids play a fundamental rôle in metabolism. The function of proteins is determined by the specific amino acids they contain and their position in the molecule. The amino acids are the precursors of several hormones and probably of some vitamins. They play a dominant part in the conjugation mechanisms which are known as detoxication or defense processes.

Glycine is readily synthesized by the mammalian organism and is available in relatively large amounts. It has an important part in the synthesis of creatine. It is extensively employed for the conjugation of aromatic acids. In the latter reaction part of the glycine is derived from exogenous sources and the remainder is synthesized. The conjugation of benzoic acid

with glycine has been developed into a successful test of liver function. The coupling of glycine with nicotinic acid, a vitamin, presents an interesting reaction, the purpose of which is not understood. The excretion of uric acid is influenced by glycine and by the drugs conjugated with the amino acid.

Ornithine is used by the bird to conjugate aromatic acids. In the mammal it is a part of the Krebs-Henseleit cycle which is regarded as the mechanism whereby urea is synthesized. The comparative physiology of the fowl and mammal in regard to these reactions presents promising clues concerning certain phases of intermediary metabolism.

Cystine is used for the formation of taurine, glutathione and the mercapturic acids. The latter reactions appear to be the initial means employed by the organism to metabolize the unsubstituted aromatic ring.

Glutamine is employed only by man and the chimpanzee for conjugation with phenylacetic acid. Glutamine appears, however, to play a more important part in metabolism than this solitary reaction indicates. Glucuronic acid and the ethereal sulfates are not directly dependent upon amino acids, but may be derived from them.

There is good evidence that thyroxine, epinephrine, histamine and, perhaps, other hormones are derived from amino acids.

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Chapter IX

Metabolism of Proteins and Amino Acids

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Theodore Weyl

Born in Berlin, Germany, in 1851 and died in 1913. He was first to obtain alanine from protein and discovered a color reaction for creatinine. He was an authority on hygiene and the author of several books.

Introduction. Protoplasm sans protein does not exist. Every living cell contains protein and this type of material is involved in all vital phenomena. Skin, hair, tendon, and muscle are chiefly protein in nature. Even the lipid-rich nervous tissue and the inorganic salt-rich osseous tissue contain large amounts of characteristic protein substance. The list can be extended to include all the tissues of the body. Moreover, all the enzymes and some of the hormones elaborated in the animal organism are proteins.

The proteins themselves are all made up of small building units, the common amino acids. Although a single protein molecule may contain hundreds or even thousands of these simple building blocks, little more

than a score of different kinds of amino acids have been found present in the animal or plant proteins thus far examined. That this number of amino acids makes possible the existence of an infinite number of different kinds of protein molecules is not surprising. The amino-acid composition of any one protein may differ qualitatively and quantitatively from that of another. Thus, to our knowledge insulin contains less than a dozen different amino acids and casein contains about a score; ovalbumin contains five per cent isoleucine and serum albumin but two per cent. Furthermore, differences in the arrangement of the amino acids in the peptide chains, in the looping of the chains, and in the nature and number of lateral bonds between chains all make possible the existence of countless distinct kinds of proteins.

It is understandable that the story of the metabolism of proteins and amino acids is not a simple one. It concerns itself not only with the synthesis and degradation of protein, but also with the fate in the body of each individual type of amino acid. A great many important details of the story remain to be elucidated. Nevertheless, the results of the efforts of numerous investigators afford an insight into many of the mechanisms involved.

Digestion. Dietary protein must be broken down to simple diffusible units before it is of value to the body. This is efficiently accomplished within a few hours with the help of a number of different proteolytic enzymes. Although no breakdown of food protein occurs in the mouth, the physical effects of mastication and admixture with saliva facilitate subsequent digestion in the stomach. Here the strongly acidic gastric juice with its content of the powerful proteolytic enzyme, pepsin, converts most proteins to metaproteins, proteoses, and peptones. Rennin in gastric juice has a high milk-clotting activity and helps to prevent the casein of fluid milk from passing too quickly through the stomach. The protein in the clot is subjected to peptic action.

After leaving the stomach through the pylorus, the protein material of the chyme is subjected to the action of a number of proteolytic ferments in the small intestine. Trypsin and chymotrypsin — which are transported to the duodenum from the pancreas as zymogens by the pancreatic juice — fragment proteic substances of the chyme to proteoses, peptones, and polypeptides. The action of these two pancreatic enzymes is not, however, identical with that of pepsin. The digestive effect of pepsin, trypsin, and chymotrypsin is additive. The partially digested products are finally hydrolyzed to amino acids by peptidases of pancreatic juice, intestinal juice, and the intestinal mucosa. In the absence of the stomach, native food proteins can be completely digested by proteolytic ferments in the small intestine, despite the lack of preliminary peptic action.

The action of proteolytic enzymes in the gastro-intestinal tract can be duplicated with enzymes *in vitro*. The conditions for complete digestion in this case are not always as favorable as they are *in vivo*, however, for the

digestive end products are not continuously removed as they are in the small intestine. Proteins may be similarly hydrolyzed *in vitro* by boiling with acid or alkali. Appropriately supplemented mixtures of amino acids which have been prepared by enzymatic or acid hydrolysis of casein are sometimes used clinically for intravenous therapy.

Absorption. Although protein breakdown is initiated in the stomach and some diffusible products of peptic digestion may be formed, no significant absorption of these substances occurs in this organ. In the small intestine, however, the end products of protein digestion are very actively absorbed. This process is greatly facilitated by the large surface area afforded by the wall of the small intestine with its *plicae* and *villi*, and by the efficient vascular network of this section of the alimentary tract. Absorption is most rapid in the duodenum and is essentially completed by the time the intestinal contents reach the ileocaecal valve.

In the course of digestion proteins are converted almost completely to amino acids and, generally speaking, it is in the form of these simple units that they are absorbed. For the most part the amino acids are absorbed into the blood in the capillaries of the villi, although a small percentage is taken up by the lymph and reaches the blood by way of the thoracic duct. It is noteworthy that the larger fragments of partially digested protein produce toxic effects, if they are introduced into the blood stream. The enzymes in the small intestine and in the intestinal mucosa effectively split these products to simpler units, however, and thereby guard against the entry of harmful substances into the blood. Some peptides may be absorbed along with amino acids,[1] but there is normally no absorption of undigested protein. That the intestinal mucosa is sometimes permeable to protein, particularly in very young animals, is well known. Indeed, some allergic phenomena are related to the absorption of traces of undigested dietary protein.

Disposal of the Products of Protein Digestion. The amino acids are apparently not changed during absorption from the small intestine and an increase in the amino-acid content of the blood after food intake can readily be demonstrated. Some interesting information on the question of the immediate disposal of amino acids introduced into the blood is afforded by experiments of Van Slyke and Meyer, in which it was shown that injected amino acids are avidly taken up by the tissues.[2] Hepatic tissue was found to take up more per unit of weight than muscular tissue. While the amino-acid content of the muscles and kidneys remained unchanged during the three hours following the injection, the amino acids in the liver fell back almost to their original level and an equivalent amount of urea nitrogen was formed.

The ultimate fate of amino acids taken into the body is varied. Some may be broken down to provide energy, carbon dioxide, water, and ammonia which goes to form urea. Others may serve as precursors or as con-

stituents of a number of biological substances, including hormones and detoxication products; and still others may be incorporated into body protein. Amino acids resulting from the catabolism of body protein as well as those absorbed from the intestine follow these metabolic paths. More detailed information on the vicissitudes of the amino acids will be presented later.

A small amount of the end products of protein digestion may escape absorption in the small intestine and be attacked by microorganisms in the large intestine and colon. Bacterial action results in the production of typical putrefactive products, such as organic acids and amines, which are structurally related to the amino acids from which they are derived. Although some of the products formed are toxic, they do not enter the blood in significant amounts, since they are formed in a section of the alimentary tract where absorption is poor.

Deamination. The removal of the α-amino group usually represents an initial step in the catabolism of an amino acid. Three types of simple deamination are conceivable:

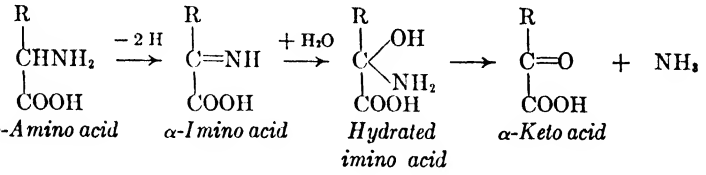
$$\begin{aligned} RCHNH_2COOH + 2\,H &\longrightarrow RCH_2COOH + NH_3 \quad \text{(reductive)} \\ RCHNH_2COOH + O &\longrightarrow RCOCOOH + NH_3 \quad \text{(oxidative)} \\ RCHNH_2COOH + HOH &\longrightarrow RCHOHCOOH + NH_3 \quad \text{(hydrolytic)} \end{aligned}$$

Reductive deamination of amino acids in the tissues apparently does not occur. Oxidative deamination is, however, commonly observed, and oxidative rather than hydrolytic deamination appears to be the usual occurrence in the animal body. Years ago it was pointed out by Dakin [3] that α-keto acids are commonly more readily oxidized in the tissues than the corresponding α-hydroxy acids, and that, in general, the fate of an amino acid and of the corresponding α-keto acid is identical, whereas the α-hydroxy acid analog, being presumably a secondary reduction product, may behave differently.

Krebs [4] in classic experiments with tissue slices has shown that amino acids are readily deaminated in the presence of oxygen with the resulting production of α-keto acids and ammonia. The rate of deamination was highest for kidney tissue, although liver and other tissues were also capable of effecting the deamination of amino acids.

Ingestion of a comparatively large amount of a particular amino acid results in the subsequent excretion in the urine of a small amount of the corresponding α-keto acid.[5] Also supporting the view that α-keto acids are normally intermediary products in the deamination of amino acids *in vivo* is the observation of du Vigneaud and Irish [6] that administration of levorotatory phenylaminobutyric acid to a dog results in urinary excretion of the acetyl derivative of the dextrorotatory form of the acid. The formation of the latter acid involves asymmetric synthesis from the corresponding α-keto acid.

An imino acid is believed to be formed as an intermediate in the process of oxidative deamination. One may picture the mechanism as follows:



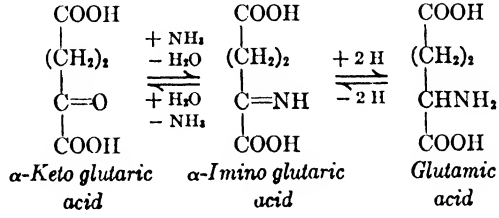
The process of oxidative deamination is, however, not always as simple as that pictured above. Indeed, the amino group need not be liberated as ammonia, but may be transferred to a keto acid by a transaminating enzyme. The details of this process will be described later.

There is evidence that β-hydroxy amino acids, under particular conditions, may be deaminated in a special manner. Thus, Chargaff and Sprinson [7] have reported the anaerobic deamination of serine by cell-free extracts from mouse, rat, and rabbit liver slices. The general reaction may be briefly formulated as follows:

$$RCHOH \cdot CH(NH_2) \cdot COOH \longrightarrow RCH_2COCOOH + NH_3$$

Amination, Including Acetylating Amination and Transamination. In the presence of ammonia and a suitable catalyst α-keto acids may be reductively aminated *in vitro*, as shown by Knoop and Oesterlin.[8] This reaction is the reverse of that of simple oxidative deamination which has already been pictured. It has long been known that α-keto acids may be similarly converted to α-amino acids by perfusion through the surviving liver.[9]

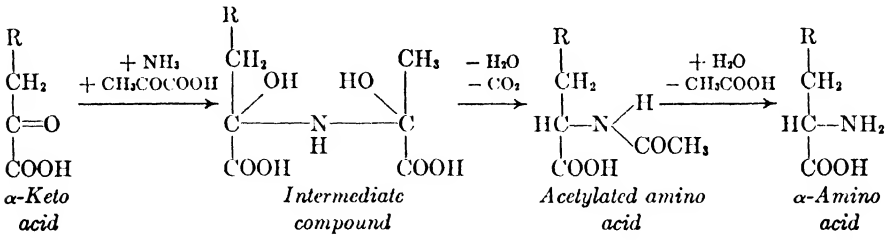
Glutamic acid is of particular interest in connection with the problem of amination *in vivo*. von Euler and his associates [10] have demonstrated that a specific enzyme occurs in liver that is capable — in the presence of the appropriate co-enzyme — of dehydrogenating glutamic acid, with the ultimate production of α-ketoglutaric acid and ammonia. This reaction is, moreover, reversible:



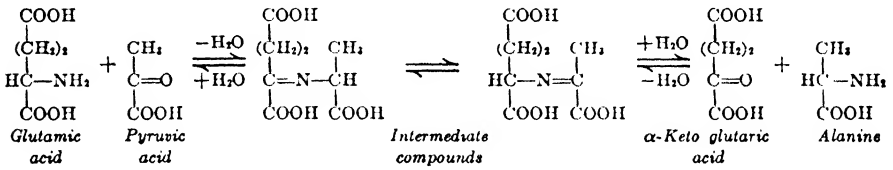
The hydrogen for the synthesis of the amino acid from the imino compound is supplied by the appropriate coenzyme.

Ingeniously planned metabolic experiments of du Vigneaud and Irish [6] have lent strong support to the acetyl theory of Knoop. According to

this conception, the amination of an amino acid from an α-keto acid may be accomplished by a mechanism in which the acetyl derivative of the amino acid plays a part. A coupled oxidation-reduction involving pyruvic acid and ammonia takes place. The probable steps in the amination are shown below:

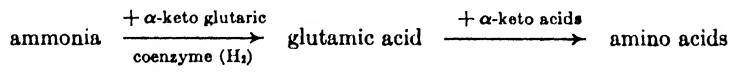


Ammonia is not invariably associated with the amination of α-keto acids. Amination may, indeed, occur in the absence of this base by a process of transamination in which the interaction of a keto acid and an amino acid is involved. This important phenomenon was demonstrated by Braunstein and Kritzmann [11] and it may take place in many tissues. A typical transamination reaction is pictured below.



One of the interacting compounds in this process must be either glutamic acid or aspartic acid. It is not clear to what extent other amino acids may be involved in transamination. According to one concept most amino acids may participate. On the other hand Cohen,[12] who worked with preparations of the enzyme transaminase, has reported that relatively few substances can take part in this reaction with the dicarboxylic compounds. In this connection it is possible that the activity of the isolated enzyme system is not as extensive as that of the intact tissue. In any case, transamination is a mechanism of considerable importance in connection with the problem of amination and deamination *in vivo*.

von Euler and his associates [10] have suggested that the synthesis of glutamic acid from ammonia and α-keto glutaric acid is an initial step in the synthesis of amino acids in the animal organism. This function was portrayed by them as follows:



In passing, it might be mentioned that glutamic acid or aspartic acid reacts with citrulline, under aerobic conditions and in the presence of kid-

ney slices, to yield arginine [13] — a process which has been termed transimination, because of the formation of the imino group of arginine.

The importance of the dicarboxylic amino acids, glutamic acid and aspartic acid, in amination mechanism is noteworthy. It is of interest also that oxidatively deaminated residues of these amino acids, α-ketoglutaric acid and oxaloacetic acid, may be involved in carbohydrate metabolism.[14] Here we have another example of a link between the metabolism of protein and the metabolism of carbohydrate.

Formation of Urea. Urea is the chief nitrogenous end product of protein metabolism in mammals, as well as in amphibia and elasmobranch fishes. A variety of nitrogenous compounds serve a similar purpose in other animals — uric acid, for example, in birds and reptiles (other than the turtle, which excretes urea) and ammonia in teleost fishes. The goosefish (*Lophius piscatorius*) excretes large amounts of trimethylamine oxide in the urine.[15] The non-toxicity, solubility, and diffusibility of urea render it admirably suited for its rôle as a metabolic end product.

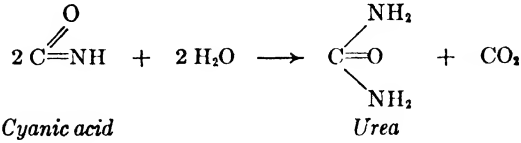
As early as 1882 it was shown that urea could be formed in the liver.[16] This organ is, indeed, the sole site of urea formation in some, if not in all, mammals. The classic experiments of Bollman, Mann, and McGath [17] with hepatectomized dogs showed conclusively that in this animal, urea formation takes place only in the liver. In agreement with the observations of these investigators on dogs is the report that severe liver damage in a human individual may, before death, result in a great suppression of urea synthesis, accompanied by an accumulation of amino acids in the blood.[18] That the liver is not the sole site of urea formation in all animals, however, is exemplified by the findings of Kirsch, who reported extra hepatic urea production in selacians.[19]

The bulk of the nitrogen for urea synthesis comes from the amino nitrogen of the amino acids. A portion of the nitrogen is, however, of different origin. Thus absorbed ammonia, ammonia from deaminated purines and pyrimidines, amide nitrogen, and nitrogen in other groupings, such as that in the imidazole group, may be converted to urea.

The mechanism of urea formation has long been the subject of investigation. Among the older theories may be mentioned the dehydration theory, involving the intermediate formation of ammonium carbamate, and the oxidation theory, involving the intermediate production of cyanic acid. According to the first concept, the formation of urea was pictured as follows:

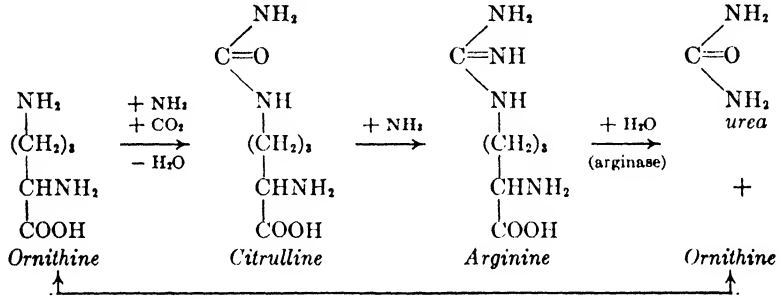
$$CO_2 + H_2O + 2\,NH_3 \longrightarrow \underset{\text{Ammonium carbonate}}{C(=O)(ONH_4)_2} \xrightarrow{-H_2O} \underset{\text{Ammonium carbamate}}{C(=O)(NH_2)(ONH_4)} \xrightarrow{-H_2O} \underset{\text{Urea}}{C(=O)(NH_2)_2}$$

According to the cyanic acid theory, cyanic acid is formed as a result of oxidation and cleavage of amino acids in peptide linkage. The acid is converted to urea by hydrolysis:



Cyanic acid *Urea*

A more modern theory proposed by Krebs and Henseleit [20] on the basis of *in vitro* studies with liver slices describes a cyclic process of urea formation which involves the participation of a number of substances, including arginine, citrulline, ornithine, and arginase, as well as carbon dioxide and ammonia. This hypothesis has enjoyed wide popularity. The reactions thought to be involved are shown below:

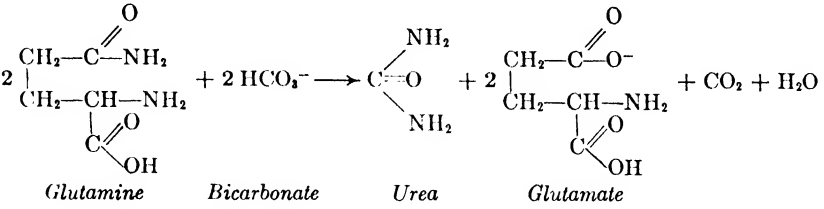


The process described above is continuous; the ornithine formed as a result of the action of arginase on arginine may be used again and again. Addition of ornithine to the reaction mixture in liver-slice experiments was found to accelerate the rate of urea production from ammonia. More recently, it has been shown with the aid of isotopes that carbon dioxide is taken up in the process of urea formation.[21] Ornithine also catalyzes the formation of urea from ammonia in the perfused liver.[22]

The story of the formation of urea in the body is apparently not identical, however, with the process pictured in the attractive cycle of Krebs and and Henseleit. While many experimental observations may be interpreted as supporting the Krebs-Henseleit cycle, other observations are available which do not support this mechanism of urea formation. The significance of citrulline in the ornithine cycle, for example, is not clear in view of the fact that the mechanism whereby citrulline is rapidly converted to arginine is absent from the liver, but is present in the kidney.[13] Moreover, in experiments with the perfused rat liver, citrulline was found to have no catalytic effect on urea formation, and it was not converted into arginine or ornithine.[22] On thermodynamic grounds alone Borsook and Dubnoff [23] have pointed out that even if the ornithine cycle is correct, as far as it goes, it is incomplete. The formation of urea from ammonia and carbon dioxide under physiological conditions entails a gain of almost 14,000 calories of

free energy per mole of urea and reactions yielding the requisite amount of free energy must therefore participate in the process. It is unlikely that carbon dioxide and ammonia enter the cycle as such.

It is noteworthy that a mechanism of urea synthesis involving glutamine but not ornithine has been described by Leuthardt.[24] The following equation illustrates this mechanism.



The presence of glutamine in blood has recently been demonstrated.[25]

Bach [26] has proposed a scheme of urea formation which connects the mechanism of Krebs and Henseleit with that of Leuthardt. Bach's conception was not, however, supported by the experimental results of Trowell,[22] who studied urea formation in the perfused rat liver.

It may be concluded that the complete story of the formation of urea *in vivo* remains to be told. When the normal mechanism of urea formation is finally elucidated, however, it may well be found to include details of the mechanisms described by Krebs and Henseleit and by Leuthardt.

Specific Dynamic Action of Proteins and Amino Acids. After the ingestion of food the metabolic rate is increased above the basal level. This stimulating effect on the heat output of the animal body has been commonly called specific dynamic action — a term originated by Rubner. The calorigenic effect of dietary protein is particularly pronounced. While dietary carbohydrate and fat also exhibit this phenomenon, they do so to a much more limited degree than protein.

As an illustration of the calorigenic effect of protein, one may describe the observation of Williams, Riche, and Lusk [27] on the heat production of a dog before and after ingestion of 1200 grams of meat. The heat production of the animal under basal conditions was approximately 23 calories per hour. One hour after the feeding it rose to 36, two hours later to 42 calories. It remained at an elevated level for several hours and then gradually diminished, until after 21 hours it was approximately 25 calories. For every 100 calories contained in the protein of 1200 grams of meat, 130 calories were produced — a 30 per cent excess.

Not only protein, but also a hydrolyzate of protein as well as individual amino acids have been reported to be capable of producing a calorigenic effect. In the latter connection phenylalanine, glycine, alanine, tyrosine, leucine, histidine, glutamic acid, and other amino acids have been specifically credited with producing a specific dynamic action. The negative findings of an early investigator for a particular amino acid (*e.g.*, glutamic acid)

have sometimes been followed by a positive finding for the same amino acid in more recent studies.[28, 29]

Specific dynamic action attains its maximum value in animals at environmental temperatures above 25° C; at low temperatures, 0–5° C, the fasting energy production is already nearly the resting maximum.[30]

Many explanations for the calorigenic action of proteins and amino acids have been considered. The work of digestion and absorption apparently plays no part in specific dynamic action, for an orally effective amino acid is also effective when administered parenterally. The liver plays a rôle in the phenomenon, for the administration of glycine or alanine to the hepatectomized dog produces no calorigenic effect.[31]

A few years ago, after careful consideration of the then available information, Borsook [30] pointed out that there were two factors which might account for the increase in energy formation after the ingestion of protein or amino acids. One factor is approximately constant and represents the increased energy production attending the metabolism and excretion of the nitrogen; the other, more variable, factor is related to the utilization of the non-nitrogen moiety of the amino acids. In connection with the hypothesis that amino acids behave as cell stimulants — a conception which in the past was popular in relation to explanations of specific dynamic action — Borsook, after reviewing the evidence, concluded that amino acids do not act as primary stimulants to cellular metabolism.

More recently, after studies with a number of amino acids, Kriss [29] pointed out that his own findings are consistent with the theory that the specific dynamic effects of amino acids are by-products of intermediary chemical reactions. Furthermore, these effects could be most closely correlated with the energy resulting from the metabolism of the amino acids.

Amino Acids and Carbohydrate Formation. If an animal is poisoned with phlorizin a condition is produced which is known as phlorizin diabetes, in which the renal threshold for sugar is lowered and glucose is excreted in the urine. Such animals have been used extensively in studying the problem of amino acids and carbohydrate formation in the animal body. Any orally administered or parenterally injected glucose is excreted in the urine by these animals. The feeding of proteins has likewise been observed to effect an increased glucose excretion by phlorizinized dogs. Indeed, feeding experiments with different proteins have been interpreted as indicating, for example, that 48 per cent of casein, 55 per cent of serum albumin, 65 per cent of gelatin, and 80 per cent of gliadin may be converted to glucose in the animal body.[32] Starving phlorizinized animals have been reported, on occasion, to have a dextrose-to-nitrogen ratio in the urine of approximately 3.65 to 1, which might be interpreted to indicate that about 58 per cent of the body protein was convertible to glucose.*

* The D : N ratio in phlorizinized dogs is not always the same, however, and various values have been reported by different investigators. It is of interest that a completely diabetic individual has been reported to have a D : N ratio of 3.65 to 1.[33]

Individual amino acids as well as proteins have been tested with respect to their ability to effect an excretion of extra glucose by phlorizinized experimental animals. The experiments of Dakin [34] in particular have provided information in this regard and have permitted compilation of lists of so-called "glycogenic" and "non-glycogenic" amino acids. Some of the former amino acids were seemingly converted carbon for carbon into glucose; others, such as glutamic acid and aspartic acid, produced glucose equivalent to but three of their carbon atoms.

Information comparable to the foregoing has been obtained with the help of entirely different techniques. Thus the administration of some amino acids to fasted animals, like the administration of glucose, effects a subsequent increase in the glycogen content of the liver. In another method of assessment of the carbohydrate-like action of particular amino acids, advantage is taken of the observations of Butts and his associates,[35] who have found that the administration of some amino acids, like the administration of glucose, alleviates experimental ketonuria in rats.

A summary of the results of studies reported in the literature on the carbohydrate-like effect of amino acids as ascertained by different methods is shown below.

Carbohydrate-like Effect of Amino Acids *

Amino Acid	Extra urinary glucose in phlorizinized dog	Hepatic glycogen deposition in fasted rat †	Reduction of experimental ketonuria in rat
Alanine	+ *dl*	+ *l*, *d*, *dl*	+ *l*, *dl*
Arginine	+ *l*	+ *l*	+ *l*
Aspartic acid	+ *dl*	+ *l*, *dl*	+ *l*, *dl*
Cysteine	+ *l*	− *l*	
Cystine		− *l*	−
Glycine	+	+	+
Glutamic acid	+ *l*	+ *l*, *dl*	+ *l*
Histidine	+ (?) *l*	+ *l*	+ *l*
Isoleucine	− *dl*	+ (slight) *dl*	− *dl*
Leucine	− *dl*	− *dl*	− *dl*
Lysine	− *l*	− *l*, *dl*	
Methionine	+ *dl*	− *dl*	
Norleucine	+ *l*, *d*, *dl*	+ *dl*	+ *dl*
Phenylalanine	− *dl*	+ *dl*	+ *dl*
Proline	+ *l*		
Serine	+ *dl*	+ *dl*	
Threonine		+ *dl*	+ *dl*
Tryptophane	− *l*	− *l*, *d*, *dl*	+ *l*, *d*, *dl*
Tyrosine	− *l*	+ *l*	− *dl*
Valine	+ *l*, *d*, *dl*	+ *dl*	+ *dl*

* The letters in the table refer to the configurational series and not to the observed rotation.

† In this respect, for one reason or another there are conflicting reports for a few amino acids. In these instances, the effect listed in the table is what was considered to be the most representative finding.

While the results obtained with different techniques commonly agree, it is evident from the table that, in certain instances, an amino acid may have a positive effect as measured by one method and a negative effect as measured by another. This has led to some confusion, since published lists of "glycogenic" amino acids have not generally, if at all, provided adequate information to serve as a basis of classification.

It is often assumed that the extra glucose excreted when a "glycogenic" amino acid is administered to a phlorizinized animal represents a product of the direct conversion of the deaminated moiety of the amino acid. Similarly, it might be considered that the glycogen deposited after feeding "glycogenic" amino acids represents the chemically converted amino acid. The results might also be interpreted as indicating that the administered amino acids themselves are not converted to glucose or glycogen, but that they have a sparing action which permits the conversion to carbohydrate of some metabolite (derived perhaps from tissue protein) in the body.

Long-sought information on this question has been afforded by experiments with isotopically labelled amino acids. Olsen and his associates [36] administered glycine containing heavy carbon to mice whose liver glycogen was subsequently analyzed for the carbon isotope. In these experiments the hepatic glycogen was found to contain only a small excess of the isotopic "tag" and the increase in liver glycogen was far more than could be accounted for by the conversion of labelled glycine to glycogen. Only one of the "tagged" carboxyl carbon atoms of the administered glycine was to be found for every four or five molecules of glucose in the glycogen. Comparable results were obtained in an earlier study by Gurin and Wilson [37] in experiments with phlorizinized animals. These investigators showed that the extra glucose excreted in the urine after the administration of "tagged" alanine contained little excess isotope. The bulk of the carbohydrate formed in these representative cases did not originate by direct conversion of the administered amino acid but rather by the conversion to carbohydrate of some metabolite, which itself was in some way spared by the amino acid administered.

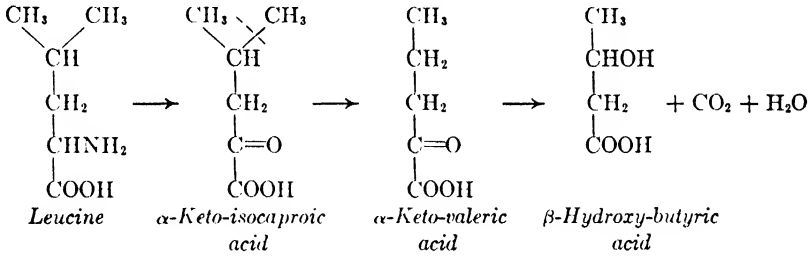
It is noteworthy that the injection of a "glycogenic" amino acid (glycine) into the blood of hepatectomized dogs does not alleviate the hypoglycemia.[38] The production of carbohydrate following the administration of certain amino acids to experimental animals is a phenomenon in which the liver apparently plays a rôle.

Amino Acids and Acetone Body Formation. The apparent conversion of certain amino acids to acetone bodies may be demonstrated in a number of ways. An amino acid in a perfusing fluid may be passed, for example, through a surviving liver and the presence or absence of acetone bodies in the perfusate may be determined. In this manner the formation of acetone bodies has been demonstrated in experiments in which leucine, phenylalanine, and tyrosine were employed.[39] The feeding of an amino acid to a

diabetic individual or experimental animal may also provide information on the problem of amino acids and acetone body production. Thus, Baer and Blum [40] found that when either leucine, phenylalanine, or tyrosine was fed to a diabetic patient there was an increased excretion of β-hydroxybutyric acid in the urine. Leucine has also been shown to be ketogenic by Cohen [41] in an extensive study in which the tissue-slice technique was applied. Butts and his co-workers have studied the same problem by feeding particular amino acids to fasted rats and subsequently determining the urinary acetone body excretion. Racemic leucine, for example, was shown to be ketogenic by this technique.[42]

Of the amino acids present in protein, only a few — leucine, phenylalanine, and tyrosine — are commonly considered to be ketogenic. Under certain conditions *dl*-isoleucine may apparently also yield acetone bodies in the rat.[42] In addition, it has been reported that hydroxyproline is considerably ketogenic in liver-slice experiments.[43]

The conversion of one of the ketogenic amino acids, leucine, to β-hydroxybutyric acid may be pictured as follows:



In analogous fashion one may depict the formation of ketone bodies from other ketogenic amino acids. Experiments with amino acids labelled with isotopic carbon are necessary, however, before one can definitely state to what extent the carbon chain of an administered amino acid may, under particular experimental conditions, be actually converted *in vivo* to acetone bodies.

Biocatalysts and Protein Metabolism. Enzymes, vitamins, and hormones are associated, in one way or another, with the metabolism of proteins and amino acids. All the isolated enzymes are protein in nature and their synthesis in the body is part of the kaleidoscopic picture of protein metabolism. Enzymes themselves are involved in the synthesis and breakdown *in vivo* of proteins as well as of carbohydrates and fats. It is not feasible to discuss here the enzymes that are known to be directly associated with the metabolism of proteins and amino acids. It will suffice to say that these biocatalysts are involved in the processes of synthesis, transformation, and degradation which characterize the metabolism of amino acids and proteins.

Vitamins are associated with the story of protein metabolism in that many of these dietary essentials are incorporated *in vivo* into the prosthetic

group or coenzyme of certain enzymes which, in turn, are involved in metabolic processes in the organism. To cite a single example, thiamine pyrophosphate is the coenzyme of carboxylase.[44] The co-carboxylase-carboxylase enzyme system is directly associated with the metabolism of pyruvic acid, an important intermediate of both protein and carbohydrate metabolism.

Many of the hormones, like insulin, are protein or protein-like in nature. Moreover, epinephrine and thyroxine are closely related chemically to tyrosine, an amino-acid structural unit of proteins. These chemical relationships alone would make certain hormones a part of the story of protein metabolism. The interrelation is, however, much more complex and extensive than that of mere structural affinity. The effect of thyroxine in increasing the metabolic rate is an example of a general effect of a hormone on metabolism. One may likewise cite the growth factor of the anterior pituitary as being involved in protein metabolism, for this hormone promotes the retention of nitrogen.[45] Furthermore, adrenal steroids appear to be associated with the process of hepatic glycogen formation from protein metabolites.[46]

Our knowledge of the relationship between the biocatalysts and protein metabolism is still fragmentary. Many phases of this problem remain to be investigated.

Dynamic State of Body Protein. It was long supposed that anabolic processes, such as the synthesis of protein in the animal organism, were largely restricted to the extent of replacing the losses of "wear and tear" — losses which were reflected by the endogenous quota of Folin. Our concepts of protein metabolism, have, however, undergone revision in recent years. Several years ago Borsook and Keighley,[47] on the basis of their own experiments and information available in the literature, suggested that the breakdown of intracellular protein is continuously in progress even in an animal in nitrogen equilibrium. This breakdown bears no "wear and tear" connotation, but is directly proportional to the level at which the nitrogen metabolism has been set by previous dietary history. According to this concept, a large part of the amino acids from ingested protein is synthesized into peptides and protein, while the remainder is catabolized — the nitrogen appearing in the urine. The anabolic process may account for 50 per cent or more of the nitrogen intake. In nitrogen balance, the synthetic and degradative reactions offset each other.

Final proof of the falsity of the conception that tissue proteins, once laid down, remained essentially unchanged until eventually destroyed by the "wear and tear" of metabolism was afforded by the brilliant work of Schoenheimer and his colleagues,[48] who studied the problem of protein metabolism with the aid of amino acids labelled with isotopes. Nitrogen (N^{14}) in amino acids was replaced with its heavy atomic analog (N^{15}) and, in some experiments, the carbon chain of an amino acid was labelled by

replacing attached hydrogen atoms with deuterium, the heavy isotope of hydrogen. These isotopic "tags" permit tracing in the body of the groups to which they are attached as effectively as if the latter bore miniature red lanterns.

When a labelled amino acid such as leucine or glycine was fed to rats, it was found that only a part of the dietary nitrogen could be located in the urine.[49, 50] Determination of the isotope (N^{15}) content of protein in different parts of the animal body revealed that much of the isotope was incorporated therein. The protein of the serum and of the internal organs appeared to be most active in accepting dietary nitrogen; muscle and skin were less active.[49] Owing to the large amount of muscle and skin, however, these tissues had the greatest share in the uptake of dietary nitrogen. In contrast to the plasma proteins, hemoglobin exchanges N^{15} slowly.[51]

Not only is labelled dietary amino nitrogen incorporated in the tissue proteins, but the rest of the amino acid may be incorporated as well. The leucine isolated from the tissues of rats fed this amino acid with deuterium "tags" attached to the carbon chain, for example, was found to contain deuterium. Furthermore, when labelled amino acids such as leucine (N^{15}) and glycine (N^{15}) were fed to rats and a number of amino acids later isolated from the tissues of the animals, it was found that not only the isolated glycine and leucine, but almost all the other isolated amino acids contained isotopic nitrogen. All the amino acids isolated, other than lysine, were apparently capable *in vivo* of continuously interchanging groups containing nitrogen. The dicarboxylic acids are particularly active in this process.

It is apparent that the peptide bonds linking amino acids in tissue proteins are not rigid and fixed — being broken only when the protein molecule disintegrates — but are being continually broken and re-formed. As pointed out by Schoenheimer,[48] the uptake of nitrogen is a rapid process and it follows that the opening and closing of peptide bonds must be a fast reaction. In the liver and intestinal mucosa, for example, more than half the protein apparently may be broken down and resynthesized in ten days.[23] The turnover in the muscles is less rapid and it is even less in the erythrocytes.[23]

Available experimental data on the replenishment of plasma protein in dogs that have had this protein depleted by plasmapheresis have led Madden and Whipple [52] to conclude that a dynamic equilibrium exists between the protein of the plasma and a portion of the cell and tissue body protein. With regard to the give and take between body protein and plasma protein, it is of interest to note the theory of Schmidt, Allen, and Tarver [53] that the transformation of existing proteins into others may take place in the body without an intermediate breakdown to the amino-acid stage.

A modern concept of protein metabolism (cf. Borsook and Keighley; Schoenheimer) pictures a continuing and dynamic exchange between tissue proteins and substances in a metabolic pool. This pool is made up of a

mixture of substances — indistinguishable as to origin — which normally represent not only compounds derived from catabolic processes in the tissues but also materials absorbed from the intestine. The metabolic reactions are enzymatic in nature. Although a balance between them is maintained, the synthetic and degradative reactions are different for, as pointed out by Borsook and Keighley, the physiologic steady state between protein and amino acids in tissues is far from a thermodynamic equilibrium. All regeneration reactions entailing an increase in free energy must be coupled with another process. Schoenheimer[48] has stated that the synthetic processes are coupled with chemical reactions such as oxidation or dephosphorization. When oxidation systems fail, as in death, the synthetic processes also cease and the continuing degradative reactions lead to the dissolution of the thermodynamically unstable structural elements.

Question of Protein Storage. It has long been known that the feeding of protein to a fasted experimental animal results in an increase in hepatic protein.[54] The protein enrichment of the liver which follows the ingestion of a diet high in protein is associated with an increased content of protein per unit weight of tissue, as well as with hyperplasia or hypertrophy.[55] The liver, however, is not the only organ which is influenced by administration of a diet rich in protein. Addis and his co-workers [56] have studied the increase in the protein content of various tissues of animals fed different amounts of protein in the diet. As the protein intake was increased, these investigators noted that the proportion allocated to the liver, kidney, and blood serum increased, while there was a slight fall in the proportion allocated to the carcass.

On fasting, the quantities of protein lost by various organs and tissues of the body vary considerably. Thus, during a seven-day fast in rats, the liver loses 40 per cent of its protein, the alimentary tract 28 per cent, the kidney 20 per cent, drawn blood 20 per cent, and the heart 18 per cent, as compared to losses of 8 per cent for muscle, skin, and skeletal protein, 5 per cent for brain, and 0 per cent for eyes, testicles, and adrenals.[57]

It has not been found possible to single out, on a chemical basis, any particular "reserve" or "storage" protein. Thus, Luck [55] has shown that the liver proteins, including albumin, euglobulin, and pseudoglobulin, participate equally in the function of storage. Borsook and Dubnoff [23] in discussing the question of "labile" nitrogen or "reserve" protein have pointed out that the "labile" nitrogen may not be distinguished primarily by a difference in composition but by its location. The liver, kidney, and intestines change quickly in size and protein content with changes in the level of protein alimentation. In the course of a fast the "labile" protein nitrogen would arise at first from the substance of the more labile organs. On prolonging the fast, the lower limits of size of these organs would be approached and larger amounts of nitrogen would come from structures such as the muscles, which are slower to change. A part of the liberated muscle

nitrogen, according to this view, would be resynthesized into liver and kidney protein; the remainder would participate in maintaining the characteristic and constant composition of free amino acids in the blood and tissues, and eventually would be catabolized.

It is worthy of note that the liver of a protein-depleted animal is very susceptible to injury by a variety of toxic agents. To cite an example, fifteen to twenty minutes of chloroform anesthesia are frequently fatal to protein-depleted dogs, while comparable non-depleted animals tolerate ninety minutes of anesthesia with little injury to the liver.[58]

On the basis of data from studies of the regeneration of plasma protein in dogs depleted of protein by plasmapheresis (bleeding with return of red cells suspended in an appropriate solution), Madden and Whipple [52] believe that a "steady state" or "ebb and flow" exists between plasma protein and a portion of cell and tissue body protein. When plasma protein is depleted, replacement is possible up to a certain limit by "reserve stores" of protein in the organs. Madden and Whipple [52] estimate that normal dogs have sufficient materials in storage to form a quantity of plasma protein one to two times that normally present in their circulation.

In the special sense that the animal body, which is well nourished with regard to protein, is protected against some types of adversity (depletion of plasma protein, hepatoxic agents, etc.) as compared to that of a protein-depleted animal, it may be said that the former possesses a "reserve" of protein. This qualifiedly termed "reserve" is not, however, distinguishable chemically from the rest of the body protein. Indeed, it is an integral part of the latter and participates, like body protein in general, in "continuing" metabolism.

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Chapter X

Intermediary Metabolism of Individual Amino Acids

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Emil Fischer

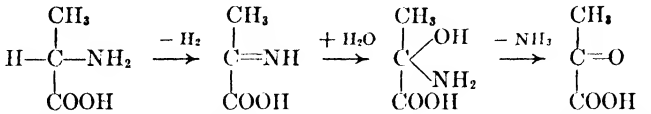
Born in Euskirchen on the Rhine in 1852 and died in 1919. Fischer was the most versatile organic chemist of his time. He and his students developed methods of synthesis for a number of amino acids, sugars, purines, and iodine-containing fatty acids. He orginated the peptide-linkage theory of protein structure.

Introduction. Our knowledge of the intermediary metabolism of each of the more than twenty common amino acids forms an integral part of the story of the metabolism of protein. In the foregoing chapter, discussion was devoted largely to phases of protein metabolism that involve proteins and amino acids in general. In the following pages amino acids will be considered more or less individually to facilitate discussion of particular features of their metabolism. The optically active amino acids mentioned in the text are all of the naturally occurring *levo* stereochemical configuration, unless otherwise designated.

Alanine and Glycine. Alanine and glycine are the two simplest amino acids derived from proteins. Of the two, alanine is the more typical amino

acid, since its α-carbon atom is asymmetric. Glycine, on the other hand, is the only common amino acid which is optically inactive.

Alanine is readily deaminated *in vivo* and *in vitro*. The oxidative deamination may be represented as follows:



The ammonia formed *in vivo* goes to form urea and the pyruvic acid may be oxidized to yield CO_2, H_2O, and energy. Other amino acids are believed to undergo a similar process of deamination.

The pyruvic acid formed as a result of the oxidative deamination of alanine is also an intermediate in carbohydrate breakdown, and it therefore represents an example of the interrelationship between the metabolism of protein and that of carbohydrate.

A study of the question of the conversion of alanine to carbohydrate in phlorizinized rats has been made by Gurin and Wilson [1] with the aid of isotopically tagged alanine. The carbohydrate excreted in the urine after the administration of alanine containing the heavy isotope of carbon was found to contain only a small excess of the marked element. Thus, most of the carbohydrate did not come directly from the administered amino acid, but probably from some metabolite which was "spared" by the alanine.

Alanine can be synthesized in the animal organism and it is generally present in proteins throughout the body.

Despite the simplicity of its chemical structure our knowledge of the metabolism of glycine is least clear of any of the amino acids. Experiments of Krebs [2] in 1933 indicated that the deamination of glycine could be effected *in vitro* by kidney slices, although the rate of deamination was lower than that of some other amino acids. Later this investigator reported that glycine was not deaminated by a deaminase prepared from acetone-dried pig kidney.[3] More recently, it was reported by Bach [4] that there is little, if any, deamination when glycine is incubated with slices of kidney, liver, spleen, diaphragm, or brain of the rat. Nevertheless, administered glycine can readily yield its amino group *in vivo*, as shown by experiments with glycine bearing isotopically tagged nitrogen,[5] and it is reasonable to suppose that the deamination is oxidative, as is the case with other amino acids. The question is not settled, however, and some glycine may be catabolized over a different route. In this connection it is interesting to note the observations of Kohn,[6] who reported that some glycine can be decarboxylated to form methylamine in the surviving canine liver.

Apart from its occurrence as a structural unit in proteins throughout the body, glycine performs a number of functions in the animal organism.

Its important role in detoxication is taken up elsewhere in this book. It was in the course of detoxication studies that Quick [7] determined the rate of synthesis of glycine *in vivo*. This synthesis in a human individual may amount to 0.55–0.70 gram per hour.

Glycine is one of the precursors of glycocholic acid, the salts of which are important constituents of bile. Experiments in which glycine was labelled with an isotope have shown that this simple amino acid is also a precursor of the amino-acid moiety of the creatine molecule.[8]

The opinion that glycine (or gelatin which contains about 26 per cent of this amino acid) can increase the muscular strength of normal individuals was probably based on the chemical relationship of glycine to creatine, the important muscle extractive, as well as on early favorable reports in the literature. More recent research on this question has indicated that glycine and gelatin possess no unique value in increasing muscular strength.[9]

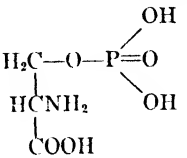
Hydroxy Amino Acids. Formerly four hydroxy amino acids were commonly considered constituents of proteins: serine, threonine, hydroxyglutamic acid, and hydroxyproline. Hydroxyproline is heterocyclic in nature and will be discussed later in this chapter along with other heterocyclic amino acids. β-hydroxyglutamic acid is no longer considered a constituent of the protein molecule. (See Chapter I.)

We know nothing concerning the metabolism of hydroxylysine, an amino acid whose presence in protein was confirmed comparatively recently by Van Slyke and his associates.[10] Our information on the metabolism of the acyclic hydroxy amino acids is restricted to that gained from studies of serine and threonine.

The metabolism of serine, the β-hydroxy derivative of alanine, has recently been studied by means of the isotopic tag technique. Experiments of Stetten in which *dl*-serine containing the heavy isotope of nitrogen (N^{15}) was fed to rats and the location of the isotope in the tissues was subsequently determined indicate that dietary serine can be incorporated as such into proteins and phospholipids of the organs.[11] In this connection it may be recalled that phosphatidylserine is a normal component of certain cephalins.[12]

The experimental results of Stetten also indicate that serine can be decarboxylated *in vivo* to form ethanolamine. Furthermore, the high isotope content of the cystine isolated from the organ protein of rats fed isotopic serine indicates, according to Stetten, that the carbon chain of serine can be converted to the carbon chain of cystine in the animal body.

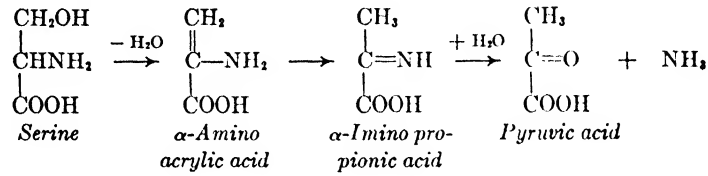
It is noteworthy that phosphoserine occurs in phosphoproteins.[13, 14]



Phosphoserine

Much investigative work remains to be carried out on the relationship between the metabolism of phosphorus and that of serine.

Like most other amino acids, *dl*-serine may be deaminated under aerobic conditions *in vitro* by kidney slices.[2] Of especial interest is the report that the deamination of serine may also be accomplished in a biological system under anaerobic conditions.[15] Chargaff and Sprinson [15] have formulated the anaerobic deamination as follows:



dl-Serine can be deaminated in this fashion not only by certain microorganisms but also by cell-free extracts from mouse, rat, and rabbit livers.

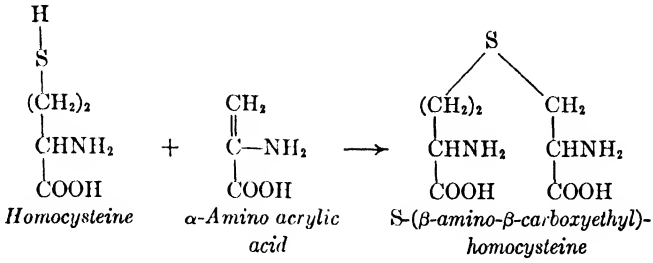
Our knowledge of the metabolism of threonine is fragmentary. The demand for threonine in the animal body, unlike that for serine, cannot be met by *in vivo* synthesis.[16] It has been demonstrated in experiments with rats that the administration of *dl*-threonine may be followed by an increase in liver glycogen.[17] This amino acid also decreases the experimental ketonuria produced in rats by feeding butyric acid.[17]

The anaerobic deamination of threonine, like that of serine, has been observed in some biological systems.[15] α-Ketobutyric acid is obtained as a deamination product of threonine under these conditions.[15] However, the relationship of this mechanism to the deamination of α-hydroxy amino acids *in vivo* remains to be determined.

Sulfur-containing Amino Acids. The need of the body for sulfur is met by the sulfur-containing amino acids of dietary protein. Elemental or inorganic sulfur is of little use to the animal organism, but the sulfur of methionine, cystine, or cysteine is readily available. The sulfur-containing amino acids are structural constituents of body protein. In addition, they serve as precursors of physiologically important compounds and are involved in detoxication mechanisms *in vivo*.

Methionine may be converted to cystine in the body, but the reverse is not true. Details of the process are meager, but a number of studies, including experiments in which an isotopic tag was employed, have demonstrated the conversion of methionine sulfur to cystine sulfur.[18] The relationship of serine to cystine [11] has already been mentioned in the preceding section on hydroxy amino acids.

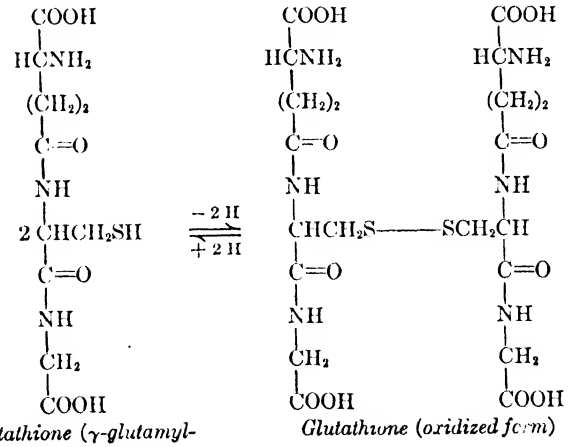
In the conversion of methionine sulfur to cysteine sulfur it seems probable that S-(β-amino-β-carboxyethyl)-homocysteine is formed as an intermediate compound, as suggested by Brand and his associates.[19] These investigators pictured homocysteine reacting with α-amino acrylic acid as follows:



Homocysteine — *α-Amino acrylic acid* — *S-(β-amino-β-carboxyethyl)-homocysteine*

The thiol ether presumably may then be split to yield cysteine as one of the reaction products. It is now known that amino acrylic acid may be formed as a result of the anaerobic deamination of serine.[15] Two molecules of cysteine may be dehydrogenated to yield one molecule of cystine, a disulfide bond being formed.

The reductive splitting of disulfide bonds and the reversal of this reaction are of importance in oxidation-reduction phenomena in the body. The physiologically important peptide, glutathione contains cysteine in its reduced form. On oxidation the cysteine is converted to cystine and two molecules of the tripeptide are joined in disulfide linkage. The reversible reaction is shown below:



Glutathione (*γ-glutamyl-cysteinyl-glycine*) — *Glutathione* (*oxidized form*)

It is noteworthy that many enzymes, *e.g.*, cathepsin and papain, are activated by substances containing the sulfhydryl group. On the other hand, in a protein hormone such as insulin (which contains cystine) reduction of disulfide linkages and the appearance of sulfhydryl groups entails a loss of physiological activity.

Like other amino acids, methionine, cysteine, and cystine can be broken down in the body to yield, ultimately, carbon dioxide, water and ammonia. The sulfur of the amino acids is largely oxidized to sulfate and excreted in the urine. Some sulfate may be used for detoxication purposes in the body.

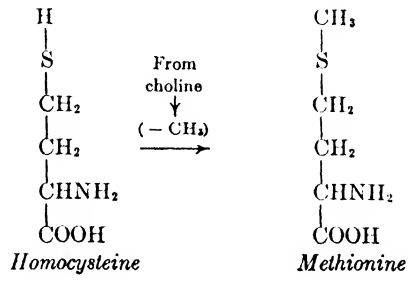
Presumably an initial step in the degradative process consists in oxidative deamination. For methionine, at least, there is evidence that this may occur. The keto acid analog of methionine may be formed from this amino acid *in vivo* [20] as well as *in vitro*,[21] and is capable of replacing dietary methionine for growth purposes in the young rat.[22]

The sulfur-containing amino acids are also involved in detoxication mechanisms. Their role in this connection is discussed in Chapter 8.

There is evidence that methionine as well as cysteine may serve as a precursor to taurine. After feeding tagged methionine to dogs, taurine containing the isotopic sulfur could be isolated from the animal's bile.[24]

Methionine, which contains a "labile methyl" group, takes part in transmethylation reactions *in vivo*. That methylation could occur in the animal body has been known for a long time. As early as 1887 there were indications that pyridine could be detoxified by methylation.[25] It was not until the past few years, however, that the interesting role of methylation in metabolic processes was revealed. Owing to the research activity of a number of investigators, the importance of the so-called labile methyl group — a chemical radical which is labile in the sense that it can, if necessary, be transferred *in vivo* from one organic compound to another — is now well appreciated.

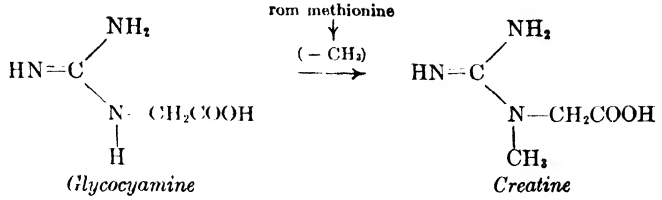
The demethylated product of methionine, homocysteine (or its disulfide analog, homocystine), is not capable of replacing dietary methionine in supporting the growth of young rats. Normal growth is made possible, however, if choline is also included in the otherwise deficient ration. Under these conditions methionine may apparently be synthesized *in vivo* by the transfer of a methyl group from choline to homocysteine.[26]



Homocysteine *Methionine*

Other compounds including lecithin, phosphorylcholine, and betaine, like choline, are able to act as methyl donors under these conditions.[27]

The methyl group of methionine may be employed in the synthesis of other compounds in the animal body. Experiments in which deuterium was used as an isotopic tag have clearly demonstrated that the methyl group of methionine may be utilized in the *in vivo* synthesis of both choline and creatine.[28] Interestingly, creatine is readily formed by liver slices from glycocyamine in the presence of methionine.[29]



Glycocyamine *Creatine*

The methylation of creatine is irreversible. Indeed, the methyl group of creatine, unlike comparable groups in choline, betaine, and methionine, is not transferable *in vivo*.

The degeneration of the kidneys, which is observed in rats fed a diet deficient in sources of the labile methyl group, may be prevented by the inclusion in the diet of methionine, choline, or betaine.[30] It is also worthy of note that the accumulation of fat which occurs in the liver of experimental animals under particular dietary conditions may be counteracted by substances, such as methionine [31] or choline,[32] that contain a biologically transferable methyl group.

It is of interest that some compounds, such as the betaine of cystine and of alanine, are lipotropic,[33, 34] but do not promote the growth of young mammals fed a diet containing homocystine but lacking in methionine.[cf. 27] Furthermore, not all substances containing a methyl group are lipotropic. To cite but two examples, ergothioneine (the betaine of thiolhistidine) [35] and abrine (amino-N-methyltryptophane) [36] are not effective lipotropic agents.

Valine and the Leucines. Valine, leucine, isoleucine, and norleucine may be catabolized in the animal organism to provide carbon dioxide, water and ammonia. With regard to their structural function, one may state that three of these amino acids, *i.e.*, valine, leucine, and isoleucine, are common units of tissue proteins. Little is known, however, of the distribution in the animal body of norleucine, although it has been isolated from nervous tissue.[37] The need of the animal organism for norleucine may be wholly met by *in vivo* synthesis; this is not the case for the other three amino acids discussed here.[16]

When valine is fed to a phlorizinized dog, an amount of extra glucose equivalent to three of the five carbons of the administered compound may be demonstrated in the urine.[38] It has, moreover, been shown in experimental studies with rats that the administration of *dl*-valine may result in a deposition of hepatic glycogen.[39] Administration of *dl*-valine has also been reported to decrease the excretion of acetone bodies in rats suffering from experimental ketosis.[39] Studies with liver slices also provide evidence of the antiketogenic effect of *dl*-valine.[40]

Leucine does not effect the excretion of a significant amount of extra glucose in the urine of a phlorizinized dog.[41] Furthermore, *dl*-leucine does not promote glycogen formation in appropriate test animals.[42] On the

other hand, acetone bodies are formed when this amino acid is perfused through the surviving liver.[43] Similarly, when fed to a diabetic patient, leucine also effects an increase in the urinary excretion of β-hydroxybutyric acid.[44] Butts and his co-workers [42] have shown that *dl*-leucine may contribute to acetone body formation in the rat.

Under appropriate experimental conditions *dl*-norleucine is antiketogenic and may also give rise to an appreciable amount of liver glycogen in the rat.[42] Similarly, the administration of norleucine to a phlorizinized dog effects an excretion of extra glucose in the animal's urine.[45]

dl-Isoleucine has been reported to yield neither extra glucose nor a significant amount of acetone bodies when fed to the phlorizinized dog.[46] Acetone bodies are sometimes found, however, when isoleucine is perfused through the liver.[47] Butts and his associates [42] also report that *dl*-isoleucine may give rise to acetone bodies when administered as the sodium salt to fasted rats. These research workers also observed that the administration of *dl*-isoleucine to fasted rats may be followed by a deposition of glycogen in the liver. The metabolism of this amino acid is indeed interesting. On one hand, under certain experimental conditions, *dl*-isoleucine may increase the excretion of acetone bodies in rats; on the other hand its administration may be followed by carbohydrate deposition in the liver. This difference in behavior may depend on the demethylation or deethylation of the amino acid in the course of its metabolism.

Dicarboxylic Amino Acids. It is only within recent years that some of the interesting metabolic roles of aspartic acid and of glutamic acid have been elucidated. These acids are important structural units of tissue proteins and are capable of being synthesized in the animal organism. One of the substances under discussion, glutamic acid, is also a constituent of glutathione, a tripeptide of physiological interest, particularly in connection with oxidation-reduction phenomena in the animal body. Waelsch and Rittenberg,[48] who studied the metabolism of the tripeptide with the aid of isotopes, have suggested that glutathione may act as an intermediate between free amino acids and proteins.

Like other amino acids, glutamic and aspartic acids may be oxidized in the animal body to provide carbon dioxide, water, and energy. The amino nitrogen liberated is ultimately converted to urea and excreted.

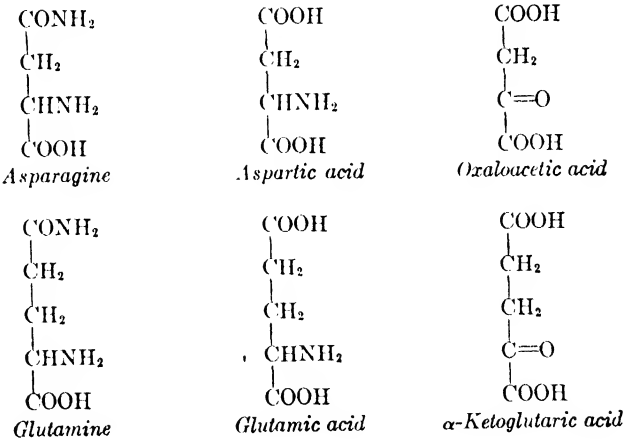
The keto acids formed as a result of oxidative deamination of glutamic and aspartic acids are of unusual interest. Both of these substances, like their parent amino acids, are intimately associated with the phenomenon of transamination — a process which was discussed in the preceding chapter. The keto acid derived from aspartic acid, oxaloacetic acid, plays a fundamental role in cellular oxidations.[49] Similarly, it has been postulated that both α-ketoglutaric and oxaloacetic acids play a part in the intermediary metabolism of carbohydrates.[50] In this connection it is noteworthy that α-ketoglutaric acid can be synthesized from pyruvic acid in

the pigeon liver by a mechanism involving the participation of carbon dioxide.[51] It is believed that the carbon dioxide combines directly with pyruvic acid to yield oxaloacetic acid, the latter then combining with an additional molecule of pyruvate to form α-ketoglutarate.[51]

Both aspartic acid and glutamic acid effect an excretion of extra urinary glucose when they are fed to a phlorizinized animal.[52] In these instances, however, the amount of extra glucose excreted is equivalent to only three carbon atoms of each of the administered dicarboxylic acids. Hepatic glycogen deposition follows the administration of either glutamic acid or aspartic acid to fasted rats.[53]

The amides of the dicarboxylic amino acids are worthy of note, since these acids often occur in this form in nature. The amide of glutamic acid, glutamine, is involved in a mechanism of urea formation distinct from the ornithine cycle, according to Leuthardt.[54] This mechanism has already been considered in connection with the discussion of urea formation in the preceding chapter. The amide of aspartic acid, asparagine, may participate in a similar mechanism.[54] Glutamine also plays a role in detoxication.

The chemical relationship between the dicarboxylic amino acids and their related keto acids and amides is shown below.



Of unusual interest is the recent report of Van Slyke and his associates [55] that the bulk of urinary ammonia has its origin in the glutamine of the blood. This important finding directly relates glutamine to mechanisms for preserving the acid-base balance in the animal body. In *in vitro* studies Krebs [56] has shown that glutamine may be formed from ammonium glutamate by tissue from kidney, brain, and retina. Furthermore, extracts from tissues which synthesize glutamine contain a specific enzyme (glutaminase) which can convert glutamine to ammonium glutamate.[56]

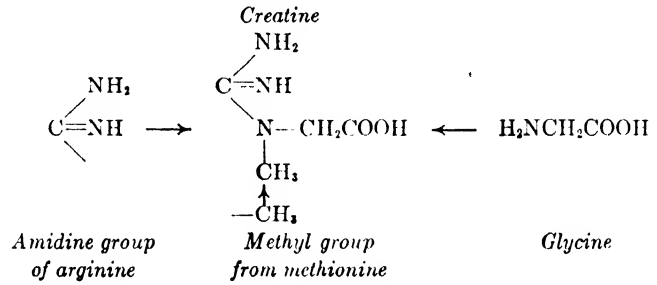
Hexone Bases. The basic amino acids, arginine, lysine, and histidine, which all contain six carbon atoms, are common structural units of tissue

proteins. Their prominence in this connection is suggested by the fact that Kossel [57] once postulated that all proteins contained a nucleus of basic amino acids.

The hexone bases are not synthesized in the growing mammal at a rate commensurate with the requirements of normal growth.[16] This does not mean that no synthesis of any of the three amino acids occurs. Synthesis of arginine by the rat, for instance, has been demonstrated, although the rate of formation was inadequate for normal growth of the animal.[58] Some synthesis of histidine may also occur in the mammalian organism, since this amino acid is not required for the maintenance of nitrogen balance in man.[59]

Arginine is readily split by arginase, an enzyme present in the mammalian liver, to yield ornithine and urea. Both arginine and ornithine are prominently featured in the mechanism of urea formation proposed by Krebs and Henseleit.[60] This mechanism is discussed in the preceding chapter.

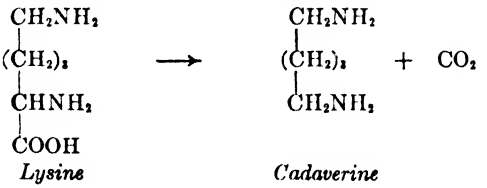
Arginine, like glycine and the methyl group of methionine, is one of the precursors of creatine in the body.[61] The origin of the various parts of the creatine molecule may be pictured as follows:



Both ornithine and arginine, when fed to a phlorizinized dog, effect an excretion of extra glucose in the urine.[46]

Lysine, according to Felix and Naka,[62] is not deaminated *in vitro* by rat kidney or liver tissues. Nevertheless, lysine can yield its nitrogen for the formation of other amino acids *in vivo*.[63] Once deaminated, however, it cannot be regenerated by amination.[63] Lysine is unique among the amino acids in this respect. It is conceivable that the α-keto group of the oxidatively deaminated residue of lysine reacts with the terminal amino group to form a substituted piperidine, and that the ring closure precludes reformation of lysine by amination.

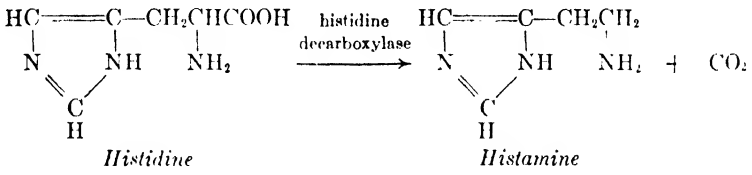
Lysine is the mother substance of cadaverine:



Ornithine, on decarboxylation, yields a comparable diamine $NH_2CH_2(CH_2)_2CH_2NH_2$, putrescine. Both cadaverine and putrescine are ptomaines and may be formed on putrefaction of protein material. Frequently, the term "ptomaine" poisoning is erroneously applied to food poisoning resulting from bacterial contamination of food. Cadaverine and putrescine have, sometimes, been observed in the feces and urine of cystinuric individuals, and in cholera the stools seem to contain these ptomaines frequently.[64] According to Wells,[64] the toxicity of both putrescine and cadaverine is slight.

Histidine can be oxidatively deaminated *in vitro* by kidney slices.[2] It may, however, be degraded *in vivo* in a different fashion. Thus histidase, an enzyme present in the mammalian liver, is capable of splitting the imidazole ring of histidine with the formation of ammonia as one of the reaction products.[65] According to Edlbacher and Kraus,[66] glutamic acid may eventually be formed from the carbon chain of histidine split in this manner.

Another enzyme, histidine decarboxylase, which is present in liver tissue as well as in the tissue of other organs, is capable of effecting the decarboxylation of histidine.



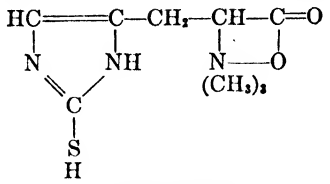
Histidine *Histamine*

Histamine, a substance of considerable physiological interest, is, perhaps, identical with the hormone gastrin. Histamine may be destroyed *in vivo* by the enzyme histaminase.

Urocanic acid (imidazolyl-acrylic acid) has been isolated from the urine of experimental animals fed histidine. Darby and Lewis,[67] who extensively studied the relationship of urocanic acid to the intermediary metabolism of histidine, have concluded that this acid is not a quantitatively important intermediate in the normal metabolism of histidine. Histidine has sometimes been considered to be one of the precursors of purines in the body. Evidence proving this suggested relationship is lacking.

Histidine is a common constituent of normal pregnancy urine, but its presence cannot be taken as a criterion of pregnancy for it is excreted in widely varying amounts by both males and females in health and in disease.[68]

Of interest in connection with histidine is the occurrence in blood of the betaine of thiolhistidine and of ergothioneine.



Ergothioneine

Nothing is known of the function of this compound in the body. It has been reported that in rat's blood, ergothioneine is exogenous and that its level in the blood of these animals is influenced by the diet.[69] No consistent effect of various types of diet on the ergothioneine content of the blood could be demonstrated in preliminary studies on human subjects.[70]

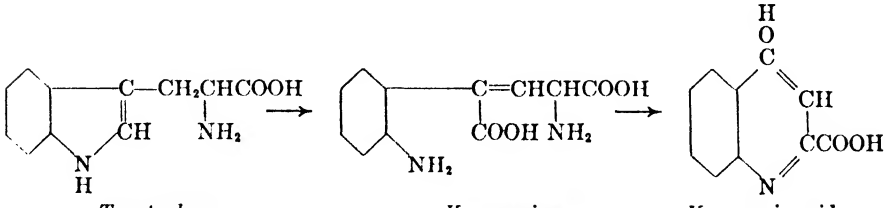
Also of interest is the occurrence in muscle of carnosine, a peptide of histidine and β-alanine, and of anserine, a methyl derivative of carnosine.

Heterocyclic Amino Acids. Four heterocyclic amino acids, tryptophane, proline, hydroxyproline, and histidine, are known to serve as structural constituents of proteins. Histidine has already been discussed.

Tryptophane may be deaminated *in vitro* under aerobic conditions by kidney slices.[2] Presumably, the catabolism of tryptophane *in vivo* may similarly begin by oxidative deamination.

In some animals, a mechanism exists for the conversion of tryptophane to a quinoline derivative. The rabbit and dog, to cite examples, can convert ingested tryptophane, in part, to kynurenic acid, which may be detected in the urine of these animals.[71] A precursor of kynurenic acid, kynurenine, may be similarly excreted. Kynurenic acid is apparently not an intermediate product but an end product of tryptophane metabolism.[72] In those animals which excrete the compound, the elimination of kynurenic acid is most readily demonstrable after the ingestion of relatively large amounts of tryptophane — the production of kynurenic acid occurring especially when there is an excess of tryptophane over normal metabolic requirements. Small amounts of kynurenic acid have been isolated from the urine of human subjects who ingested several grams of tryptophane.[73]

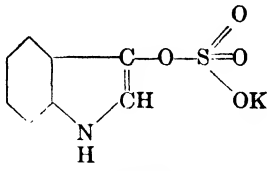
Without picturing all the postulated intermediate compounds, one may indicate the origin of kynurenic acid from tryptophane as follows:[74]



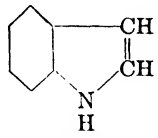
Tryptophane *Kynurenine* *Kynurenic acid*

The formation of kynurenine from tryptophane *in vivo* is not a reversible reaction for it has been shown that the former substance cannot replace dietary tryptophane.[75]

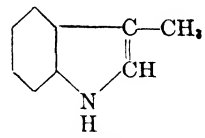
According to Kotake and Sakata, tryptophane is one of the precursors of the urinary pigment, urochrome.[76] It is also a precursor of indican, a normal constituent of urine. Indole and skatole, which are largely responsible for the characteristic odor of feces, are formed as the result of bacterial activity on this amino acid in the intestine.



Indican



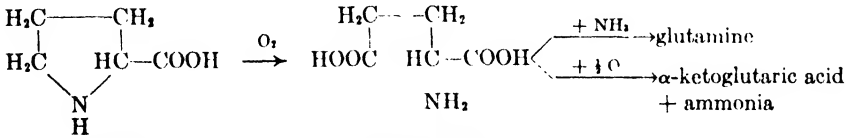
Indole



Skatole

The heterocyclic amino acids proline and hydroxyproline are capable of being synthesized by the animal organism; but *de novo* synthesis of tryptophane does not occur at all or at least not to an extent sufficient to satisfy the needs of the animal body for growth,[16] or for maintenance of nitrogen balance.[77]

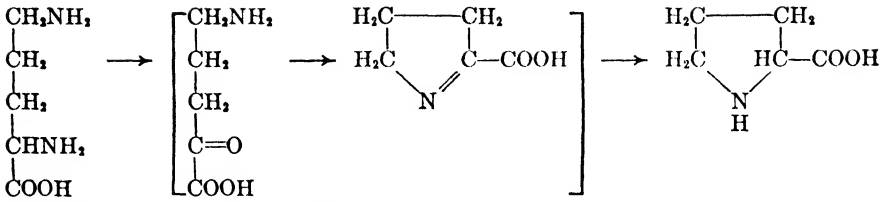
Proline does not bear a primary amino group. Deamination of this amino acid necessitates rupture of its five-membered ring. In *in vitro* experiments with kidney tissue Weil-Malherbe and Krebs[78] have observed that there is an increase in amino nitrogen during the course of the oxidation of proline. In this experiment the amino compound, itself, was not isolated but under slightly modified conditions it was possible to identify a decomposition product, α-ketoglutaric acid, and ammonia. The intermediate amino compound would appear then to be glutamic acid, and the aforementioned investigators picture the metabolism of proline in kidney as follows:



Proline *Glutamic acid*

If proline is subject to the same type of breakdown *in vivo*, then its metabolic fate in the body is similar to that of glutamic acid, the metabolism of which has already been discussed.

With regard to the origin of proline *in vivo*, it has been demonstrated in experiments in which isotopic tags were employed that ornithine may be converted to proline in the mouse. Schoenheimer[79] has formulated the conversion in the following manner:



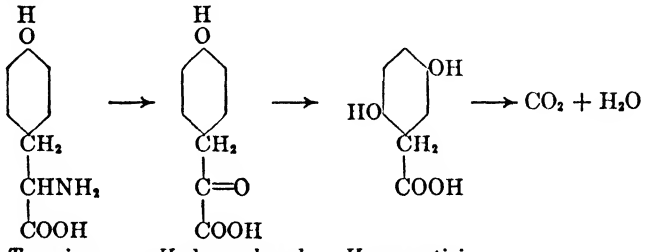
Ornithine *Probable intermediates* *Proline*

The administration of proline to a phlorizinized dog results in the excretion of extra glucose in the urine.[80]

Hydroxyproline has not been studied extensively and little is known of its metabolism. The possibility that reduction of hydroxyproline to proline is the first step in the breakdown of this heterocyclic hydroxy amino acid has been suggested.[78] This suggestion is based on the observation made in tissue-slice studies that under certain conditions hydroxyproline, like proline, causes the formation of an acid amide which reacts like glutamine.[78] It would appear from other studies, however, that proline and hydroxyproline may follow different metabolic pathways. Illustrative of this is the observation of Edson [81] that liver slices under appropriate conditions produce acetoacetic acid in the presence of hydroxyproline, whereas no significant amounts of the ketone body are formed in the presence of proline.

Aromatic Amino Acids. Phenylalanine and tyrosine are common structural units of protein. These aromatic amino acids may be deaminized by rat kidney slices under aerobic conditions,[2] and there is evidence that oxidative deamination of both substances can likewise occur *in vivo*. Like other amino acids they may be completely oxidized in the normal animal body. The aromatic amino acids have both been found to give rise to ketone bodies under certain conditions.[82] Both are considered to be ketogenic amino acids. When fed to a phlorizinized dog, neither substance evokes an excretion of extra glucose in the urine.

According to Neubauer,[83] tyrosine metabolism may have as its initial step oxidative deamination, with consequent formation of *p*-hydroxyphenyl pyruvic acid. This compound is eventually converted to homogentisic acid, which in turn is normally oxidized to carbon dioxide and water. Without including all the postulated intermediary compounds, Neubauer's scheme of tyrosine metabolism may be briefly pictured as follows:

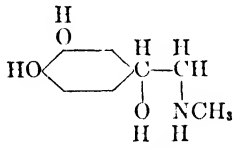


Tyrosine *p-Hydroxyphenyl-pyruvic acid* *Homogentisic acid*

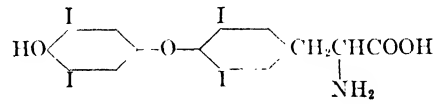
Phenylalanine, according to Neubauer, can be converted to tyrosine, and thus also plays a part in this scheme. Homogentisic acid, which appears as a metabolite in the above scheme, is excreted in the urine of individuals suffering from the inborn error of metabolism, alcaptonuria. It is readily oxidized by normal individuals.

Dakin [84] was early of the opinion that homogentisic acid was not a normal product of intermediary metabolism of tyrosine or phenylalanine, but rather that the excretion of homogentisic acid in alcaptonuric urine was the result of abnormal production and faulty destruction of this acid by individuals afflicted with this disease. In the meantime, however, excretion of homogentisic acid has been effected experimentally in man [85, 86] and in the guinea pig [86] by feeding an excessive amount of tyrosine, and in rats by feeding comparatively large amounts of phenylalanine.[87] It would therefore seem that homogentisic acid is a normal metabolite. It is normally oxidized completely, and appears in the urine of man or animals only under unusual experimental conditions when some of it escapes oxidation. Further information of interest in connection with the metabolism of tyrosine is included in the discussion of inborn errors of metabolism at the end of this chapter.

The chemical relation of tyrosine to thyroxine and epinephrine is noteworthy. It is interesting that thyroxine may be synthesized *in vitro* by the iodination of casein (which contains tyrosine) under particular experimental conditions.[88] Little is known of the details of the synthesis of thyroxine from tyrosine *in vivo*. With regard to the formation of epinephrine, it has been observed in studies with tissue slices that the decarboxylation of tyrosine may be effected by kidney tissue.[89] Conceivably, tyramine formed in this way in the kidney can be converted to epinephrine in the adrenals.[89]



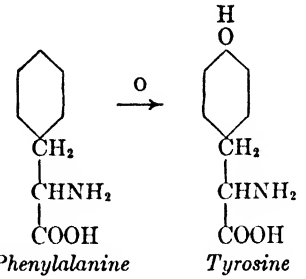
Epinephrine



Thyroxine

Tyrosine is also related to melanin, a coloring matter normally occurring in the skin. A pigment may be readily produced as a result of the action of tyrosinase on tyrosine. 3,4-Dihydroxyphenylalanine is formed as an intermediate product.[90]

dl-Phenylalanine may be converted to tyrosine by the surviving liver, as shown years ago by Embden and Baldes.[91] More recently the conversion of *dl*-phenylalanine to tyrosine in normal rats was demonstrated in experiments in which isotopically labelled phenylalanine was administered to normal rats, and tyrosine bearing the isotopic tags was isolated from the tissues.[92]



Phenylalanine *Tyrosine*

Reversal of this reaction *in vivo* apparently does not occur to any great extent, since tyrosine cannot replace dietary phenylalanine in young rats.[93]

The phenylalanine which is converted to tyrosine suffers the same fate in the body as tyrosine from any other source. Phenylalanine may, however, follow a pathway in metabolism different from that of tyrosine. The urinary picture in rabbits after ingestion of comparatively large amounts of *dl*-phenylalanine differs from that presented after ingestion of comparable amounts of tyrosine.[94] The administration of phenylalanine to individuals suffering from *Oligophrenia phenylpyruvica* increases the excretion of phenylpyruvic acid in the urine. Tyrosine has no like effect.[95] Administration of phenylalanine to an appropriate experimental animal may likewise effect an excretion of phenylpyruvic acid in the urine.[96] It is apparent that phenylalanine can be converted to phenylpyruvic acid in the animal organism. Phenylpyruvic acid, unlike phenylalanine itself or tyrosine, does not however yield acetoacetic acid in the surviving liver.[91]

There would seem to be at least two pathways for phenylalanine breakdown. In experiments in which ketone body production is observed from phenylalanine, the breakdown apparently does not proceed along the route which has phenylpyruvic acid as an intermediate.

Vitamin C exhibits an interesting relation to the metabolism of the aromatic amino acids.[97] It has been observed that *p*-hydroxyphenylpyruvic acid and homogentisic acid are excreted in the urine of vitamin C-deficient guinea pigs fed extra tyrosine. These substances do not, however, appear in the urine when an adequate amount of vitamin C is administered. Premature infants fed vitamin C-free milk likewise excrete in the urine substances derived from tyrosine.[98] Here again the administration of ascorbic acid prevents the appearance of the compounds in the urine.

Inborn Errors of Amino-acid Metabolism. Several inborn errors of amino-acid metabolism are known. The metabolism of aromatic amino acids is involved in albinism, alcaptonuria, and *Oligophrenia phenylpyruvica;* the metabolism of sulfur-containing amino acids is implicated in cystinuria. Unlike a metabolic disease such as diabetes mellitus, the inborn errors of amino-acid metabolism generally show little tendency to become aggravated as time goes on, and they are not helped by therapeutic agents. In the case of individuals with a congenital error of metabolism, a particu-

lar metabolite is not dealt with normally, owing probably to the lack or to the improper functioning of some enzyme or enzyme system. In a classic treatise Garrod [99] writes that one is tempted to regard inborn errors of metabolism as metabolic sports — chemical analogs of structural malformations.

Albinism is an inborn error of metabolism that is characterized by an abnormal deficiency in the formation of melanin, a type of pigment that plays an important role in the surface coloration of man and animals. The work of Bloch [100] affords evidence in favor of the view that the defect in albinism is a lack of the specific enzyme which is capable of producing melanin in cells that are normally sites of pigmentation. This investigator treated surviving slices of skin from man and animals with dilute solutions of various aromatic compounds and found that, when a solution of 3,4-dihydroxyphenylalanine was employed, a pigmentation of the skin resulted, except when albino hide or the white portion of the hide of spotted animals was used. That the catalyst effecting the formation of melanin is an enzyme is evidenced by the fact that it is destroyed by heat as well as by proteolytic enzymes, and is inactivated by some enzyme poisons. The amino acid 3,4-dihydroxyphenylalanine may itself be formed from tyrosine by the action of an enzyme.[90]

Alcaptonuria is an inborn error of aromatic amino-acid metabolism that is characterized by the excretion in the urine of homogentisic acid, a substance that turns dark on exposure to air and that reduces Fehling's solution. Although Wolkow and Baumann [101] in 1891 first identified the characteristic end product of alcaptonuria, the anomaly had been described centuries earlier in the medical literature. Garrod [99] describes a typical case cited by Zacutus Lusitanus in 1649. The patient was a boy who excreted "black" urine. It was thought at the time that the condition was due to charring of his bile and a course of drastic treatment was instituted which was designed to subdue the imagined fiery heat of his viscera. Despite the bleedings, purgation, baths, and drugs, the condition was not ameliorated. According to the story, the boy later married, had a large family, and lived a long and healthy life, although he continued to excrete the characteristic urine.

Alcaptonuric individuals cannot completely metabolize tyrosine and phenylalanine, and upon ingesting homogentisic acid they excrete it unchanged. Normal subjects, on the other hand, can completely metabolize reasonable amounts of tyrosine and phenylalanine.

Tissue protein as well as food protein can contribute the precursors of homogentisic acid, for alcaptonurics continue to excrete the compound when fasting. It is noteworthy, moreover, that the blood proteins as well as the hair and nails of these subjects are not deficient in phenylalanine or tyrosine. It is apparent that a portion of the amino acids is utilized normally by these individuals

Oligophrenia phenylpyruvica, one of the most recently discovered inborn errors, is a derangement of the metabolism of phenylalanine. In 1934 Folling [102] reported that the urine of certain mentally defective patients is characterized by the presence of phenylpyruvic acid. Other investigators have confirmed this observation.[103] Administration of phenylalanine effects an increase in the excretion of phenylpyruvic acid in patients with *Oligophrenia phenylpyruvica*.[95] It has been concluded that the metabolic disturbances in these individuals lies in the inhibition of the metabolism of phenylalanine at the stage of phenylpyruvic acid, the subjects being unable to oxidize this acid at a normal rate.[95]

Tyrosinosis is an error of tyrosine metabolism described by Medes.[104] On the basis of available evidence tyrosinosis should not be classified as an inborn error, since only one case has been described and it is not known if the condition is congenital. Tyrosinosis is mentioned here because it is comparable, in some respects, to an inborn metabolic error. The anomaly is characterized by the excretion of *p*-hydroxyphenylpyruvic acid in the urine. Increasing the intake of protein or the administration of tyrosine or phenylalanine results in an augmented urinary excretion of the keto acid. When the keto acid itself is fed, much of it appears unchanged in the urine. Tyrosinosis must be extremely rare. Blatherwick [105] was unable to find an additional case, although he examined 26,000 urine samples for the presence of the keto acid.

Of interest in connection with the excretion of tyrosine metabolites is the report that premature infants receiving diets relatively rich in protein may excrete *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid in the urine.[106]

Cystinuria, like other inborn errors of metabolism, is hereditary. Our knowledge of the anomaly dates from 1810 when Wollaston isolated a previously undescribed type of urinary calculus — a calculus which years later was found to be made of cystine. Cystinuria is characterized by the excretion of abnormally large amounts of cystine in the urine. The excretion continues even during fasting. Because of its insolubility, cystine may contribute to the formation of urinary concretions, and the removal of such calculi sometimes presents a complex surgical problem.[107] Paradoxically, it has been found that cystinuric individuals are able to metabolize completely the amino acid cystine when it is given orally.[108] In seeking a precursor for the cystine which occurs in the urine of cystinurics, Brand and his associates [108] studied the metabolic behavior of other sulfur compounds, including cysteine and *dl*-methionine. Both of these compounds, when given orally to a cystinuric, were found to increase the output of cystine in the urine. The work of the above investigators was, in general, confirmed by Lewis and his associates,[109] who showed, moreover, that increase in urinary cystine following oral administration of *dl*-methionine is less when the cystinuric individual is fed a high-protein diet than when he is fed a

diet containing less protein. In the latter connection, it has been suggested that the utilization of the precursor of urinary cystine by cystinurics occurs more readily under conditions of a high level of protein metabolism.[109]

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Chapter XI

Nitrogen Equilibrium and the Biological Value of Protein

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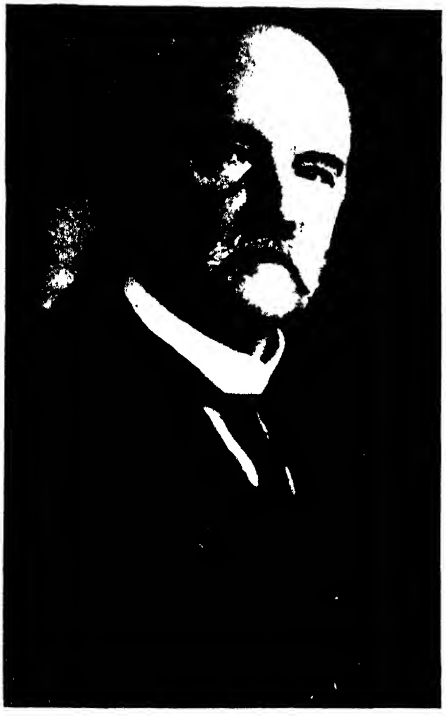


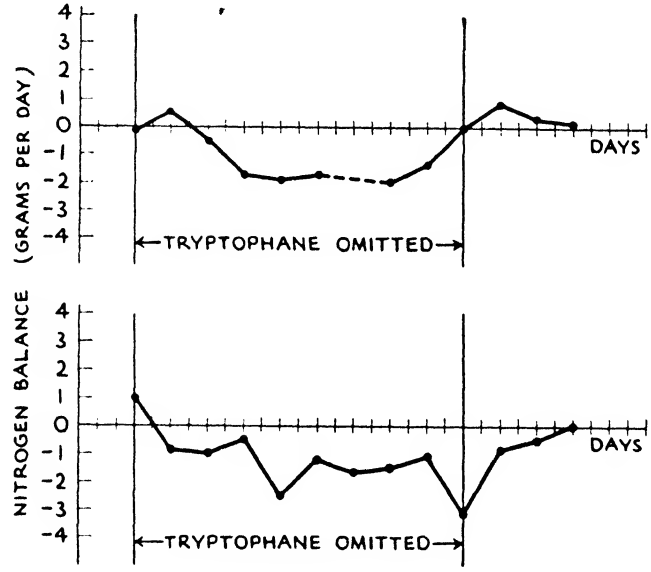
Albrecht Kossel

Born in 1853 in Rostock, Germany, and died in 1927. He was Professor of Physiology at the University of Heidelberg. As a consequence of his extensive work on the protamins, he was the first to isolate and identify histidine among the hydrolytic products. His researches on nucleic acid led him to discover adenine, thymine, cytosine, and uracil. From 1895 until the time of his death he was the editor of *Zeitschrift fur physiologische Chemie.*

In nitrogen equilibrium, the amount of nitrogen eliminated from the body over a given period of time is equivalent to that ingested in the food during the same interval. Practically all the eliminated nitrogenous compounds are to be found in the urine and feces, and in most investigations of nitrogen balance other minor channels of nitrogen excretion, such as the sweat, are not given especial attention. For some types of study it may, of course, be necessary to consider all excretory channels.

Nitrogen balance can be established at different levels of protein consumption. Thus, an adult individual who habitually eats a diet rich in protein may be in nitrogen equilibrium, although he daily consumes food containing, for example, 20 grams of nitrogen. If the same individual changes to a satisfactory diet containing only 10 grams of nitrogen in the daily portion, after a lag period, during which the nitrogen eliminated from the body gradually diminishes, a new balance can be established at the lower level of nitrogen intake. In growing individuals the mass of protein in the body is increasing and the nitrogen balance is normally positive — more nitrogen is being consumed in the food than is being eliminated. On the other hand, in starvation, malnutrition, fevers, and wasting diseases a negative nitrogen balance is the rule — more nitrogen is being excreted than consumed.

The bulk of dietary nitrogen is contributed by the proteins of the food. However, a complete mixture of amino acids is able to replace protein for the maintenance of nitrogen balance. If an essential amino acid is removed from the amino acid mixture, a negative balance ensues. Supplementation with the missing indispensable amino acid restores the nitrogen balance. An example of this phenomenon taken from a paper by Holt and his associates [1] is shown below.



Nitrogen balance in human subjects on a diet deficient in tryptophane. Redrawn from a figure by Holt *et al.*,[1] *Proc. Soc. Exp. Biol. and Med.*, **48**, 726 (1941).

This is a simple illustration of the manner in which a study of the nitrogen balance may provide information on the nutritive value of the nitrogen-containing substances in the diet. The method is, of course, more com-

monly applied to the study of dietary protein than to experimental amino-acid mixtures. The same type of information may be obtained from growth studies, a deficiency of an essential amino acid in the dietary protein preventing normal growth. It is noteworthy that the amino acids required for the normal growth of young rats, with the exception of arginine and histidine, are also required for the maintenance of nitrogen equilibrium in humans.[2] *

Years ago Mitchell [4] described the determination of the *biological value* of a protein by a method based upon nitrogen balance data obtained under definite experimental conditions. The simple calculation of the *biological value* is shown below:

$$\frac{\text{"Retained Food N"}}{\text{Absorbed Food N}} \times 100 = \textit{Biological Value}$$

Values for retained food nitrogen and absorbed food nitrogen are obtained by taking advantage of the following premises:

$$\begin{aligned}
\text{Absorbed Food N} &= \text{Food N} - \text{Fecal Food N} \\
\text{Fecal Food N} &= \text{Fecal N} - \text{"Metabolic N of Feces"} \dagger \\
\text{"Retained Food N"} &= \text{Absorbed Food N} - \text{"Excreted Food N"} \\
\text{"Excreted Food N"} &= \text{Urine N} - \text{"Endogenous N of Urine"} \ddagger
\end{aligned}$$

The complete formula for the calculation of the *biological value* of a dietary protein by this method then becomes:

$$\frac{\text{Food N} - \left(\text{Fecal N} - \text{"Metabolic N of Feces"}\right) - \left(\text{Urinary N} - \text{"Endogenous N of Urine"}\right)}{\text{Food N} - (\text{Fecal N} - \text{"Metabolic N of Feces"})} \times 100$$

This method of estimating the *biological value* of a protein is open to criticism in that it involves a determination of "excreted food nitrogen" and of "endogenous nitrogen." The distinction between "exogenous" nitrogen and "endogenous" nitrogen envisioned by Folin is no longer tenable, according to the more modern concept of protein metabolism.[5] This criticism need not, however, invalidate the estimation. As an empirical method in which the excretion of nitrogen in the urine and in the feces under the particular conditions suggested by Mitchell is determined, the procedure is capable of producing reasonable and reproducible results.

Murlin and his associates [6] have employed a method for the determination of the *biological value* of protein that is particularly useful for studies with human subjects. The procedure involves a comparison of the amount

* It has been reported that dietary arginine is essential for human adults, despite the fact that its elimination from the diet for short periods does not induce a negative nitrogen balance.[3] If this report is confirmed, it will represent a limitation of the usefulness of short-term nitrogen balance studies.

† "Metabolic N of Feces" = fecal nitrogen on a nitrogen-free diet.

‡ "Endogenous N of Urine" = total urinary nitrogen on a nitrogen-free diet.

of urinary and fecal nitrogen excreted by an individual fed an especially designed diet rich in egg protein with the amount of nitrogen excreted by the same individual fed a similar diet, in which the protein to be tested replaces the egg protein.

The *biological value* is not an absolute figure; the *biological value* of a protein fed at a 5 per cent level in the diet is, for example, greater than that of the same protein fed at a 10 per cent level.[7] It seems desirable that determinations be made at a given low level of protein intake — 5 per cent of total calories, for instance — in order to permit proper comparison of the *biological value* of one protein with that of another.

The *biological value*, moreover, does not take into account the digestibility of a protein. It provides information only on that portion of an ingested protein that is digested and absorbed. It is also desirable to know the value to the body of the whole protein as it is ingested. Mitchell has approached this problem by indicating the losses incurred by a dietary protein "in digestion" and "in metabolism" and arriving at the "net protein content" of a given food.[8] Another, and for some purposes more desirable, way of providing quantitative information on the nutritive value of a protein would be to multiply its biological value by its digestibility. The result serves as the *nutritive index* of the protein. For example, the comparative *nutritive index* of soybean protein in cooked soybeans has been determined in one laboratory to be $94.5 \times 0.90 = 85$.[9]

Inspection of the amino-acid composition of a protein is capable of providing considerable information on its nutritive value. Such examination would show, for instance, that casein is nutritionally superior to gelatin. Data on the amino-acid composition of various food proteins will undoubtedly be of even greater value in the future when more is known of the requirement of the body for particular amino acids. However, the ultimate criterion of nutritive value will always be information gained from *in vivo* studies. Such studies include those designed to determine the ability of a given protein to promote growth or to maintain nitrogen equilibrium under particular dietary conditions.

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Chapter XII

Amino Acids and Proteins in Nutrition

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Proteins in Nutrition

Synthesis of Proteins. Organisms differ in their ability to utilize nitrogen for the synthesis of proteins, essential components of all cells. Some lower forms of life can use ammonia and nitrites for this purpose, but the higher animals require amino acids. In the latter case no protein is formed if a particular amino acid is unavailable, since a cell will build its characteristic protein or none at all. Therefore, all animals must obtain these necessary amino acids either from the diet or by synthesis in their tissues.

Protein Storage. Excess fats and carbohydrates are stored in the body. In striking contrast, the amino acids appear to be utilized immediately for their normal functions or to be catabolized and burned for fuel. During the last decade there has been considerable discussion of "reserve protein," a type of protein, analogous to glycogen, for the storage of amino acids. It is apparent that there is little evidence for the presence of any protein which has as its sole function the storing of amino acids. However, an animal in the healthy state does contain more protein than one deprived of the amino acids necessary for adequate nutrition. Under these circumstances, certain protein fractions of the body such as those of blood plasma and of the liver can be readily broken down to supply nitrogen for other purposes, but when these fractions are depleted the remaining tissue proteins are tenaciously retained. In this sense, then, there may be a protein reserve.

Protein Malnutrition. Within the last few years it has become apparent that protein malnutrition is more prevalent than was originally supposed. The examination of a large number of men for the armed services has made possible the study of the incidence of dietary deficiencies in this country. The number rejected because of obvious malnutrition is small, but there are many defects which can be traced to nutritional inadequacy.[73] Youmans,[82] in a study of the rural population of Tennessee, found the incidence

of hypoproteinemia as high as 20 per cent. Investigations on 1000 adults in New York City showed that 60 per cent ate less than 42 gm of protein per day.[9] From the study of the average intake of 1000 German laborers, Carl Voit in 1881 [47] decided that about 118 gm of protein per day is required. He argued that since man is an intelligent animal and is eating this quantity, he must require this amount per day. This value is now generally considered to be higher than necessary. Moreover, the quantity of protein is not nearly so important as the "quality," which depends upon the amino acids present.

T. B. Osborne

Born in New Haven, Connecticut, in 1859 and died in 1929. He was research chemist at the Connecticut Experimental Station from 1886 to his death. Osborne was one of the foremost authorities on nutrition and the chemistry of proteins and author of numerous scientific publications. In 1910 he was elected President of the American Society of Biological Chemists.

L. B. Mendel

Born in 1872 and died in 1935. He was Professor of Physiological Chemistry at Yale. Mendel was a good teacher and a careful and painstaking scientist. He published upward of 300 papers, of which more than 100 were in collaboration with T. B. Osborne. His extensive investigations on vitamins and the rôle of lysine, tryptophane and cystine in growth are classics in the literature of nutrition.

Dispensable and Indispensable Amino Acids

In Rats. When means of separating the amino acids became available, there followed a period of intensive investigation of a great many proteins, whereby it was found that a few of them are practically devoid of certain of the amino acids. Some of these proteins are incapable of sustaining growth of experimental animals when substituted in the diet for a more complete nitrogen source. This fact was utilized in one of the first methods of investigating the importance of amino acids in nutrition. Willcock and Hopkins [88] fed zein, the protein of corn, to mice. The animals declined in weight and soon died. Addition of tryptophane greatly increased the survival time, but the mice did not grow, presumably because of the lack of some other amino acid, such as lysine.

The work of Osborne and Mendel established the importance of several of the individual amino acids. Gliadin, the protein of wheat, supported the growth of young rats only when supplemented with lysine, whereas zein required the addition of both lysine and tryptophane.[59] Such experiments furnished the first conclusive demonstration that these amino acids are required for growth. Eighteen per cent of casein in an otherwise adequate ration allows excellent weight increments in young rats; but as the amount of casein is diminished, a point is reached at which the animals no longer grow at a normal rate. These investigators found that the inclusion of cystine at this point increases weight gains.[60] Therefore, for many years, cystine was listed as an amino acid indispensable for rats. Jackson and Block [52] later demonstrated that the addition of either cystine or methionine enables the animals to grow on such a diet, and for some time the relation between these two amino acids was obscure.

Determinations of the rôle of amino acids in nutrition by feeding inadequate proteins are limited. A variation of this procedure consists of hydrolyzing the protein and removing certain amino acids by various precipitation methods. The resulting material may be incorporated as the nitrogen source in the ration. In this way, Ackroyd and Hopkins [2] fed young rats hydrolyzed casein from which the histidine and arginine had been more or less completely removed. They reported that when both compounds were absent the animals lost weight; when both were added, they grew; and when either compound was included, they only maintained their weight or made slight gains. The suggestion was made that in metabolism the two might be interconvertible. Rose and Cox [66] in the same type of experiment showed that histidine is indeed necessary for the growth of rats, but were unable to demonstrate any interchangeability with arginine.

Another method of studying the nutritional importance of the amino acids is to investigate the synthesis of these compounds. Scull and Rose [78] removed arginine as completely as possible from hydrolyzed casein and fed the arginine-free product to young rats. Litter mates were killed at the start of the experiment and analyzed for their arginine content. After

64 days the experimental animals were likewise analyzed for their content of this amino acid, and when considerable amounts of arginine were shown to have been synthesized, it was classified as a dispensable compound.

In view of the obvious limitations of the methods so far described, Rose in 1930 began investigations with diets of purified amino acids. It was immediately discovered that when a mixture of nineteen of the known protein components was incorporated in the ration of young rats as the sole source of nitrogen, rapid losses of weight occurred.[62] Moreover, the inclusion in this food of a small amount of protein caused the animals to gain,[32] a fact which Rose attributed to the presence in the protein of some hitherto unrecognized amino acid. This hypothesis was proved to be correct by the isolation and identification of an α-amino-β-hydroxybutyric acid,[53] which was named threonine because of the similarity of its configuration to that of the sugar, *d*-threose.[57] A mixture of the known amino acids, including this compound, brought about comparatively rapid gains in weight, and it was then possible to drop out individual amino acids, or groups of them, and observe the effect. By this method it was shown that valine,[67] leucine and isoleucine [92] must be present to prevent loss of weight and eventual death. The removal of tyrosine and phenylalanine produced losses in weight which the return of phenylalanine served to correct. The addition of tyrosine was not effective.[91] That there is a definite requirement for tyrosine can be demonstrated by adding the amino acid to a ration containing amounts of phenylalanine sufficient only for sub-normal growth. In this case, there is a prompt increase in the growth rate.[94] Therefore, the requirement for phenylalanine can be satisfied only by that amino acid, but the requirement for tyrosine may be filled by either of these substances. Using phenylalanine containing deuterium, Schoenheimer [58] and Rose and Howe [70] have demonstrated its direct conversion to tyrosine.

In a similar fashion, the relationship between the two sulfur-containing amino acids was solved. Surprisingly enough, it is methionine and not cystine which is the important member of this pair.[90] The absence of both leads to complete nutritive failure and death in a comparatively short time. The addition of cystine has almost no effect in modifying this trend, while methionine alone brings about rapid gains which are not improved by cystine. Here again if the essential compound is present in less than optimum amounts, the addition of cystine brings about increases in the growth rate.[93] The methionine requirement can be met only by that amino acid, but the cystine need may be satisfied by either cystine or methionine.

In reinvestigating the place of arginine in the classification of amino acids, an unexpected result was obtained. On an arginine-free diet, which could not be produced by earlier methods, it was found that the gains in weight were only 70 to 80 per cent as great as those of the controls receiving this amino acid.[63] Apparently the young rats can synthesize this com-

pound, but not at a rate rapid enough to meet the demands of normal growth. In consideration of these results, an essential amino acid is now defined as "one which cannot be synthesized by the animal organism, out of the materials *ordinarily available* at a speed commensurate with the demands for *normal* growth." [64]

In a like manner it has been shown that the removal of glycine,[54] alanine,[44] norleucine,[92] serine,[54] proline, hydroxyproline, aspartic acid, or glutamic acid [63] has no effect on the growth rate of young rats. Therefore, these compounds, along with tyrosine and cystine, are classed as dispensable.

Classification of the Amino Acids with Respect to Their Growth Effects [63]

Indispensable	Dispensable
Lysine	Glycine
Tryptophane	Alanine
Histidine	Serine
Phenylalanine	Norleucine
Leucine	Aspartic Acid
Isoleucine	Glutamic Acid
Threonine	Hydroxyglutamic Acid
Methionine	Proline
Valine	Hydroxyproline
* Arginine	Citrulline
	Tyrosine
	Cystine

It should be pointed out that although these compounds are dispensable for growth, they may well be indispensable for some other function. Indeed, if increasing amounts of benzoic acid are added to the diets of young rats, a point is reached at which the animals cannot meet the combined requirements for detoxication and growth, and the growth rate is reduced, to be resumed if a small amount of glycine is added to the ration with the benzoic acid.[43] Certain amino acids may be shown to have other functions. Under conditions of stress they may not be synthesized rapidly enough to meet the requirements of these special uses and of growth.

The nutritive value of the optical isomers of the essential amino acids has been determined by measuring their ability to supplement deficient rations. Both the *d*- and the *l*- forms of tryptophane,[13, 15, 85] histidine,[27] methionine,[51, 64] and phenylalanine [64] promote growth. On the other hand, only the natural forms of lysine,[14] valine, leucine, isoleucine [64] and threonine [87] are biologically active. The effect of the inclusion of *d*-arginine in an arginine-free diet has not yet been reported. Data obtained by substituting certain amino acids in the ration with their corresponding α-keto or α-hydroxy analog have been published. Phenylalanine, leucine, isoleucine,[64] methionine,[20, 23] tryptophane,[12, 17, 50] and histidine [28, 46] are replace-

* Arginine can be synthesized by the animal organism, but not at a sufficiently rapid rate to meet the demands of *normal* growth.

able by both their α-keto and α-hydroxy derivatives. The analogous α-hydroxy compound can be used in place of valine.[64]

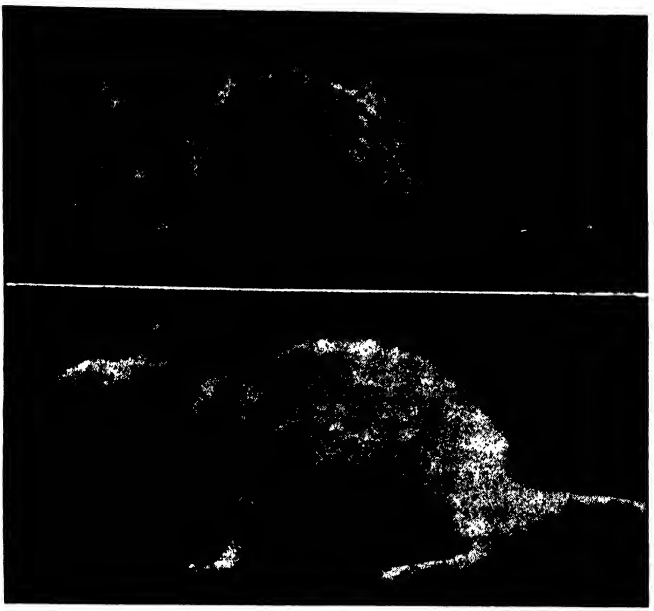
The absence from the ration of growing animals of an essential amino acid, other than arginine, results in a loss of appetite, a decline in weight and ultimate death. However, the removal of valine results in a unique set of symptoms. Rose and Eppstein found that rats deprived of valine become sensitive to touch and manifest a severe lack of coordination in movement.[67] There are reports that a tryptophane-low diet will lead to the formation of cataracts.[3, 81] In addition to the ocular lesions, which are said

FIG. 1. The initial stages of the incoordination in movement observed in animals deprived of valine.

to develop in 8 to 82 days, other changes noted in young rats were loss of weight, alopecia, greasy hair and nervousness. A detailed pathological report has been made of the changes resulting from lysine deprivation. Harris, Neuberger and Sanger[45] concluded that the cessation of growth and hypoproteinemia which they observed are due to a general inhibition of protein formation. These are not specific for lysine deficiency, but are characteristic of any animal not obtaining sufficient amino acids.

In adult animals where rate of growth can no longer be measured, the nitrogen balance is a valuable tool in determining the role of amino acids in nutrition. Investigations of the requirements of the adult rat, unfortunately, are in conflict. Wolf and Corley,[89] using nine of the amino acids essential for growth, omitting arginine, found that the animals could be maintained in nitrogen equilibrium, but that the omission of any one from the diet resulted in large nitrogen losses. They conclude that the amino acids required by the growing animal, with the exception of arginine, are also required by the adult. Burroughs, Burroughs, and Mitchell[22] found that threonine, isoleucine, valine, tryptophane and methionine are needed to replace the loss of nitrogen in endogenous catabolism in the adult rat. They report that the data suggest, but do not prove, that lysine is dispensable, an observation difficult to reconcile with the findings of Schoen-

heimer,[41] who has shown that of all the amino acids in the animal body, lysine is the only one which does not participate in the exchange of amino groups and therefore is least likely to be synthesized. Burroughs, Burroughs and Mitchell find tyrosine and phenylalanine to be interchangeable,



Courtesy Prof. W. C. Rose and Journal of Biological Chemistry

FIG. 2. Upper photograph shows a rat on the 28th day of valine deprivation. The lower photograph shows the same animal after valine had been administered for 25 days.

and leucine, histidine, and arginine to be dispensable. However, if leucine is omitted, norleucine must be included. Additional data are needed before one can choose between these two lists of amino acids essential for the adult rat.

In Mice. The amino acid requirements of the mouse have been reported recently by Bauer and Berg.[11] By feeding mixtures of the purified compounds, they have established that methionine, phenylalanine, valine, leucine, isoleucine and threonine are required for growth. Previous studies by other workers have indicated the need of this animal for tryptophane,[88] histidine [42] and probably lysine. Bauer and Berg noted no difference in the growth rate on diets with and without arginine, and conclude that the mouse is able to synthesize this amino acid.

In Chicks. Almquist and his associates [7] have investigated the qualitative and quantitative amino-acid requirements of the chick. Using diets containing casein, which is low in glycine, they have demonstrated that while the chick probably can synthesize glycine, it cannot do so at a rate which will meet the demands of early rapid growth. Increases in quantity

of glycine up to about 1 per cent give increasing rates of gain. In contrast to the rat and the mouse, the young chick seems to be unable to synthesize arginine, and loses weight rapidly on a diet low in this amino acid. Even a ration containing 20 per cent of casein does not supply enough arginine for the best growth, as is shown by the fact that the addition of arginine brings about greater gains. About 1 per cent or more of this amino acid must be furnished in the food. Using arachin, a peanut protein deficient in methionine, Almquist has shown that methionine is required and that, as in the rat, more of this amino acid is necessary if cystine is absent (0.9 per cent methionine on a cystine-free ration, or 0.5 per cent methionine in the presence of 0.4 per cent cystine). These results again demonstrate that methionine can fill the need for both methionine and cystine, but that cystine can meet the requirements only for cystine. The need for about 0.5 per cent of tryptophane was demonstrated on a ration in which the other amino acids were supplied by gelatin and acid-hydrolyzed casein. The amount of lysine furnished by 25 per cent of edestin in the ration proved to be sub-optimal, and about 0.9 per cent of this amino acid was found to be required.

In Dogs. The amino-acid requirements of adult dogs have been studied by Rose and Rice.[72] The animals can be maintained in positive nitrogen balance on the ten amino acids determined to be essential for the growing rat. Removal of arginine has no effect on the balance, so this compound is not an essential dietary component for the adult of this species. However, the removal of any one of the other nine amino acids was followed by large nitrogen losses. Thus, the amino acids essential for the maintenance of nitrogen balance in the adult dog are valine, leucine, isoleucine, phenylalanine, histidine, lysine, tryptophane, methionine and threonine.

In Man. Rose and his associates have fed mixtures of purified amino acids to healthy young men on diets otherwise almost devoid of nitrogen. By measuring nitrogen balances, it was established that the amino acids dispensable for the rat are also dispensable for the adult human.[68] Moreover, as in the dog, arginine is not needed.[65] Nitrogen losses resulted from the removal of valine, methionine,[68] threonine, leucine, isoleucine, phenylalanine,[69] tryptophane and lysine.[65] Entirely unexpected, however, is the result obtained by the removal of histidine;[69] this compound is completely dispensable in man. The amino acids required to maintain nitrogen balance are eight: threonine, valine, leucine, isoleucine, phenylalanine, lysine, tryptophane, and methionine.

Holt and co-workers,[48] feeding human subjects acid-hydrolyzed casein, which is deficient in tryptophane, find that tryptophane is an essential human dietary component, since nitrogen balance is brought about only by its addition to this ration. Deamination of a whole protein with nitrous acid is reported to destroy lysine specifically,[31] and Holt states that casein so treated and subsequently acid-hydrolyzed is unable to maintain nitrogen

balance in his adult human subjects when tryptophane is added.[4] Since he can keep his subjects from losing excess nitrogen by the addition of lysine to this mixture, he concluded that lysine is essential for the human organism. On the lysine-deficient ration, there was an increased urinary output of organic acids, and some of the subjects are said to have experienced nausea, dizziness, and hypersensitivity to metallic sounds.[5] He indicates that a diet deficient in arginine does not cause a negative nitrogen balance, but the spermatozoa are reduced in number to about one-tenth. He suggests that since sperm cells are high in arginine, an arginine-deficiency leads to atrophy of spermatogenic tissue.[49] This group of workers finds that methionine must be present in the diet in order to maintain nitrogen equilibrium. On a cystine-low ration two of their subjects were in negative nitrogen balance and they state that while the evidence is somewhat suggestive that cystine is a human dietary essential, confirmatory data from further experiments are required.[6] Rose and co-workers, as stated above, find cystine to be dispensable to the human organism.

Quantitation. After determination of the essential amino acids, it was of interest to ascertain the quantitative requirements. These amounts were established for rats by feeding them diets in which the percentage of one amino acid was increased until further increment gave no additional rise in the growth rate. The values thus obtained are shown below.[63]

Minimum Amount of Each Essential Amino Acid Necessary to Support Normal Growth When the Non-Essentials Are Included in the Food *

Amino Acid	Per Cent
Lysine	1.0
Tryptophane	0.2
Histidine	0.4
Phenylalanine	0.7
Leucine	0.8
Isoleucine	0.5
Threonine	0.5
Methionine	0.6
Valine	0.7
Arginine	0.2
	5.6

* Since the table was originally published, values for leucine and threonine have been modified to the values given here.

The quantitative requirements of the human species are under investigation by Rose and co-workers.

Functions of Amino Acids

The most important function of amino acids is the formation of tissue protein. It was formerly believed that body proteins were formed during the growing stage of the animal, and thereafter the only function of food amino acids was in maintaining and repairing tissue. Largely as the result

of the work of Schoenheimer and his associates, using molecules labeled with N^{15} and deuterium, we now know that there is complete mixing of the absorbed amino acids with those already present, and no differentiation is made between "food" amino acids and those of body proteins.[75]

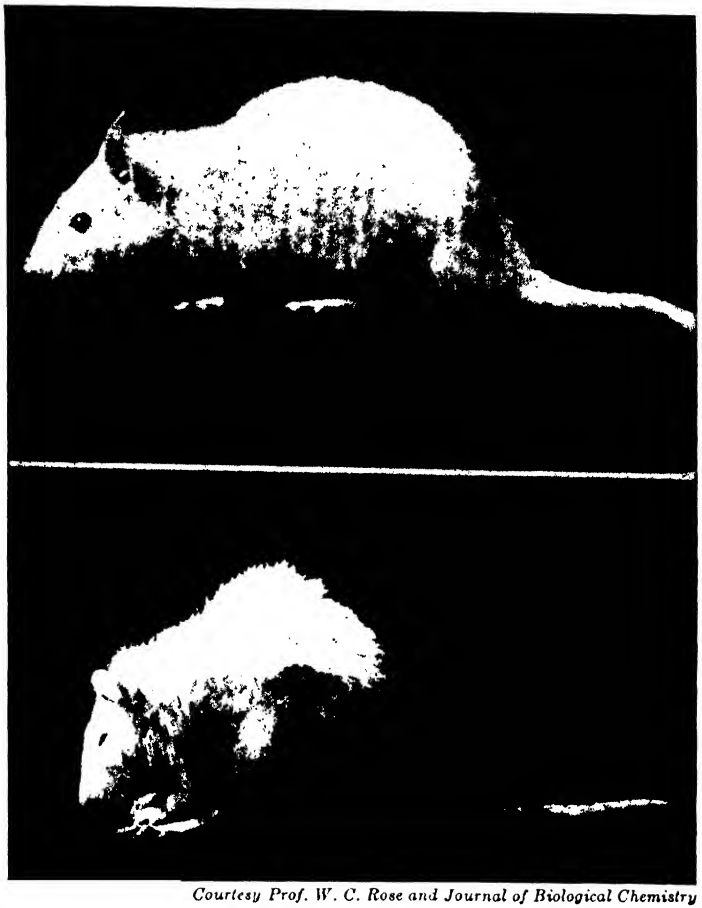
Amino acids have roles in the plant and animal body other than the formation of tissue protein. The use of glycine, glutamine, cysteine, ornithine and methionine for detoxication is discussed in Chapter 9. Another important function is the production of enzymes. Those which have been isolated in crystalline form, such as urease, pepsin, and trypsin, have been shown to be protein in nature. Other enzymes, such as Warburg's "yellow enzyme," are known to be composed of two parts, a prosthetic group, in this case riboflavin phosphate, plus a "carrier" protein.

Some of the body hormones are proteins. The most definitely characterized is insulin, which has not only been obtained in crystalline form but whose amino acid content has been carefully determined. (See Chapter 2.) Adrenaline is closely related to the amino acids tyrosine and phenylalanine. Schuler [76] showed that the kidney can decarboxylate tyrosine to tyramine, which in turn is converted into adrenaline in the adrenals.[77] Thyroxine is probably formed from tyrosine by way of diiodotyrosine in the thyroid gland.[19, 61]

Other nitrogen-containing compounds of the tissues are the so-called extractives, materials which can be removed by certain solvents. Carnosine is a dipeptide of histidine and β-alanine. Anserine is the dipeptide of β-alanine and N-methylhistidine. It has been suggested that the two compounds are formed by the decarboxylation of aspartylhistidine, but this has recently been questioned.[74] The purines and the pyrimidines are constituents of the nucleic acids which combine with proteins to form nucleoproteins, essential components of all cells. The reactions involved in the production of these compounds are still unknown, but Barnes and Schoenheimer [10] have shown that they can be synthesized by pigeons and rats from ammonia. The origin of creatine, also one of the extractives, was for many years the subject of a great controversy. This has been settled in recent years by the work of du Vigneaud,[83] Borsook and Dubnoff,[21] and Schoenheimer and his associates.[18] The amidine group of arginine reacts with glycine to form guanidinoacetic acid, which is methylated by methionine.

Some of the body lipids contain nitrogen in the form of amino acids or compounds derived from amino acids. For example, choline, a component of certain phospholipids, may be synthesized in the body by combining methyl groups from methionine [84] with ethanolamine.[79] The latter has been shown to be derived from glycine [79] or serine.[80] Phosphatidylserine, as the name implies, contains serine, while one of the components of cephalin is ethanolamine. The origin of the nitrogen base, sphingosin, a component of sphingomyelin and the cerebrosides, has not been determined.

An important role of amino acids which must not be overlooked is the formation of antibodies. It has been established that these are specifically



Courtesy Prof. W. C. Rose and Journal of Biological Chemistry

FIG. 3. Lower photograph shows a rat on a histidine-deficient diet. Upper rat received the same diet together with a histidine supplement.

modified plasma globulins, and Cannon [24, 25] has shown that hypoproteinemic animals form antibodies more slowly than normal ones. The ability of the animal to ward off disease by the production of antibodies depends on the supply of amino acids for protein synthesis.

Intravenous Alimentation

Of the amino acids present in the body, only eight must be furnished to man to maintain nitrogen balance.[65] Not only is it impossible for him to synthesize these compounds, but since he is unable to build up a store of them, a daily supply must be available. Definite quantities of these amino acids are required for ordinary protein replacement, and in all probability

larger amounts are necessary during periods of stress in which rapid protein metabolism is taking place. Ordinarily, a positive nitrogen balance is secured by a daily intake of proteins of high biological value, those containing sufficient quantities of the essential amino acids. However, occasions may arise during which an individual is unable to maintain equilibrium because of inability to obtain, retain or absorb the amino acids. Under these conditions it becomes important to find a way to make the amino acids available.

The great majority of hypoproteinemic cases which come to the attention of the medical profession are complicated by calorie, vitamin and mineral deficiencies. In most instances, a better diet will correct the situation in a short time. There are, however, certain conditions which arise not from a lack of the consumption of adequate food but from some error in its utilization. There may be defects in absorption or cases in which surgical or accidental destruction prevents the use of the gut. Allergic reactions may also be so severe that it is impossible to feed the individual adequately. In such instances some alternative route of giving nourishment must be found.

For many years the calorie requirements have been partially met by the intravenous injection of glucose solutions. This has now become a common clinical practice. Unfortunately, while the demand on the stores of the body for compounds to be burned for energy may thus be lessened, the catabolism of body proteins continues with no possibility of replacement. The intravenous administration of blood plasma or of whole blood may be of some value in such conditions. While there are reports [29, 30, 36, 86] that purified albumins of one species can be injected into other species without causing the development of allergic reactions, most biologically effective proteins appear to be antigenic. However, the nitrogen for metabolic functions *can* be supplied as amino acids, which are normal constituents of the blood stream.

Since intravenous alimentation is a technique requiring highly trained attendants, great care must be exercised in its administration. However, in cases of allergy, where the trouble lies not in absorption but in reaction to the protein itself, some other proteins to which the animal is not allergic or amino acids can be fed. Also in instances where the difficulty is in digestion of the protein, oral administration of amino acids which are directly absorbed without further alteration should be effective. Intravenous administration of amino acids is particularly important when absorption from the intestinal tract is blocked.

Within the past five years a number of investigators have studied the metabolism of amino acids in man. In Chapter 9 many of the reactions which they undergo are outlined. However, the physician has not been so much interested in the specific chemical reactions as he has been in the overall effect of the administration on the body.

After Rose and Rice [72] had determined the qualitative and approximate quantitative requirements of the adult dog, Rose, Lambert and Kade [71] set out to compare the metabolism of mixtures of purified amino acids fed or injected intravenously. It soon became apparent that the same nine amino acids which maintain a dog in nitrogen balance orally will do so when injected. Since the animal has received no nitrogen by mouth and therefore has little available, it seems reasonable to assume that the intestinal tract is not synthesizing the remaining amino acids. Also, it was conclusively shown that a dog can be maintained in nitrogen equilibrium by injection of either a mixture of the nine essential amino acids or a more complex mixture of nineteen amino acids (containing, of course, the nine essential ones). Such data prove that the animal is capable of very rapid synthesis of the non-essential amino acids. Even though the dogs received their amino acids over an extremely short period of time, only a small proportion was excreted as such in the urine, again showing that the normal animal has a great capacity for utilization of these compounds.*

These experiments with purified amino acids prove that the nitrogen requirements of an animal can be met by the amino acids themselves. The fact that an animal needs no protein as such has been established. Furthermore, it has been shown that of the amino acids known to occur in protein, only a very few cannot be synthesized by the animal.

Mention has been made of the quantitative requirements of the animal for the essential amino acids. When all these are known for man, it should be possible to devise a mixture which will maintain balance at very low nitrogen intakes. For clinical purposes, the hydrolyzate of a complete protein will act no differently from a solution of purified amino acids. The preparation is difficult to effect because an acid hydrolysis destroys tryptophane, and alkaline hydrolysis racemizes most of the amino acids. Enzymatic hydrolysis does not give complete breakdown of the proteins to amino acids and many polypeptides remain. Whether such long chains are utilized by the body is at present unknown: at least one investigator [56] feels that they can be directly incorporated into proteins. Moreover, they *may* be broken down into their components in the body and the resulting amino acids used. Nitrogen retention has been observed on intravenous administration of such hydrolyzates. However, the experiments have been of short duration, so that it is not known whether the mixture is being utilized or merely retained.

Elman [33] and Farr,[39] injecting either acid or enzyme hydrolyzates, found that there is little excretion of amino acids as such. The blood amino acids never rise greatly and always return to normal shortly after the end of the injection. The excretion of urea is higher than in protein starvation, indicating some catabolism of the administered compounds. Further information on the role of injected amino acids has been obtained by numerous

* These data will be published in full in the near future.

investigators[1, 26, 55, 87] who studied the regeneration of plasma proteins. Administering either hydrolyzates or mixtures of pure amino acids, they found that hypoproteinemic dogs will show not only large positive nitrogen balances but also increases in plasma proteins. These proteins are not produced at a rate sufficient to relieve "shock," but as Elman finds,[35] the administration will spare plasma proteins which otherwise would be used for the replacement of more essential tissues.

It should be noted that all the essential amino acids must be furnished simultaneously to obtain nitrogen retention. Berg and Rose [16] found that growth in rats on a tryptophane-free basal ration was better if the tryptophane was given at frequent intervals; Elman [34] observed that an acid hydrolyzate given by vein to dogs showed nitrogen balance only if tryptophane is added to the hydrolyzate. If the tryptophane administration is delayed by hours, a negative balance results.

There are several reports of the use of amino acids therapy in poorly healing wounds, ulcers and burns.[8] Naturally, when the nitrogen intake is low, the administration of a mixture of amino acids containing all the essential ones should improve the opportunity for protein synthesis and healing. It is difficult to understand the results, however, in patients already receiving and supposedly utilizing large quantities of protein.

Farr [38, 40] reports that children with the nephrotic syndrome have a low blood plasma amino acid concentration, and that nephrotic crises are accompanied by a sudden fall in plasma amino acids. He finds that the intravenous administration of a casein hydrolyzate to these children improves their ability to assimilate a high-protein diet and gives increased efficiency of utilization.

Thus it appears that intravenous alimentation of amino acids is entirely feasible. It has been shown that nitrogen balance can be obtained by this procedure and that plasma protein regeneration can be greatly accelerated. There are indications that other protein synthesis by the body can be aided. This is to be expected since amino acids reaching the blood stream are utilized in the same manner no matter what the source, provided that those necessary for the metabolic function of the particular cell are present.

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Chapter XIII

Utilization and Assay of Amino Acids by Microorganisms

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Louis Pasteur

INTRODUCTION

Louis Pasteur

The name of Louis Pasteur is revered and honored, not only by the people of France, because he was a citizen of France and became a national hero, but by scientists throughout the world. Pasteur was not an ordinary scientist with a limited field of endeavor. His numerous discoveries that benefited mankind were in chemistry, microbiology, bacteriology, immunology

and medicine. Pasteur was not a 40-hour-week scientist; he lived for his work and put his life and soul into it. He worked so constantly in his laboratory that he became an outstanding technician and had no use for ill-conceived, sloppily performed experiments. He was not happy sitting in an office reading reports of his associates, putting in a word here and there, and finally having the labor of others published under his name. He credited himself only for the work he actually had done.

It is indeed difficult to decide which one of Pasteur's many achievements was the most outstanding. His work is respected by the chemist for his discovery of specific rotation: the resolution of the levo- and dextro-forms of the crystals of tartaric acid. The microbiologist claims him for his refutation of the theory of spontaneous generation and for his classical work on fermentation of wine and beer. The agriculturist blesses him for his studies of silkworm disease and the protection of livestock against anthrax and other animal epidemics. The bacteriologist and the immunologist idolize him as the father of bacteriology and for his work on rabies and vaccines. Although Pasteur was not a physician, in 1873 he was elected to membership in the "Académie de Medicine." As the result of his patient, laborious and brilliant work, he gave medicine sera and vaccines -- life and hope to people of all nations. In commemoration of his name, the term "pasteurization" has been adopted to signify his unique process for the sterilization of solutions, and it is through this great discovery, which he freely gave to the world, that many milk-borne diseases and epidemics are prevented.

Considerable credit is given to Lister, the discoverer of antiseptics, but it was through Pasteur's communications on fermentation that Lister saw the possibility of keeping germs off wounds and out of the operating room.

Louis Pasteur was born December 27, 1822, at Dôle, Department of Jura, France. He was the son of a tanner. As a boy, he aspired to be an artist, and he was more interested in fishing than in studying. However, at the age of 14 he settled down and devoted himself to his studies.

After attending the Colleges at Arbois and Besançon, he entered "Ecole Normale" in Paris in 1843. It was here that his interest in chemistry was aroused as the result of attending the lectures of two well-known French scientists of that age, Balard and Dumas. It was here too that his mind began to focus on the relation between chemical structure and physical properties. During the latter part of his studies at Ecole Normale he repeated the work of de la Provostaye on the crystallization of the acids of wine. He made the interesting observation that, although the crystals of tartaric acid appear to be chemically identical in every respect, there are differences in their physical appearance. The average chemist would probably have regarded this phenomenon as one of those "quirks" of nature, but Pasteur was intrigued by the problem and worked doggedly to find the solution. He patiently examined his crystals microscopically

and, after a long search and with extraordinary care and perseverance, he separated the right- from the left-handed crystals. It occurred to him that the two crystals were the mirror image of each other. The mixture of the two types is what we now call racemic and the individual ones levo- and dextro-crystals. Pasteur made solutions of each type and of the mixture and tested them by means of polarized light. The solution of the right-handed crystals deflected the polarized light to the right, that of the left-handed crystals to the left, and that of the mixture resulted in no deviation.

The results of these studies on tartaric acid gave Pasteur recognition in France, for in 1848 he was called to the "Lycée" at Dijon as a physicist. Three months later he accepted the chair of chemistry at the University of Strassburg.

At Strassburg he pursued his studies in crystallography and racemization which won him the coveted ribbon of the Legion of Honor and a prize of 1500 francs. It was here too that Pasteur was "racemized," for he fell in love with the daughter of his "supérieur," Mademoiselle Laurent, who became not only his wife but his life-long companion and his best collaborator.

In 1854 he left Strassburg for Lille, where he was appointed Dean of the newly created Faculty of Science. Up to that time he had been concerned with investigations dealing with specific rotation and resolution of the racemic compounds into their respective levo- and dextro-forms. He had resolved amyl alcohol into the optically active and inactive fractions by esterification of the alcohol with sulfuric acid and had subsequently crystallized and separated the levo- and dextro-esters. He had also studied the conversion of quinine and cinchonine into their isomeric products.

While at Lille, he became interested in alcoholic fermentation by yeast. Lille was a prosperous town and one of its industries was the production of beer and wine, which was effected by natural processes that were difficult to comprehend. A student, whose father was worried by the many difficulties he was encountering with fermentation, was responsible for arousing Pasteur's interest in this problem which had been baffling scientists and industrialists.

At that time there were two distinct concepts concerning fermentation by yeast. Latour and Schwann had produced evidence that yeast was a living organism. Liebig, who was at the height of his popularity and eminence, opposed this view and strongly upheld the decadent hypothesis of spontaneous fermentation. In his sphere of influence Liebig's word was law, and neither the opinion of Latour and Schwann nor the evidence they presented could cause the mighty Liebig to alter his erroneous attitude. This was the situation when Pasteur, the hard-working, modest scientist, entered the controversy. It is no wonder that two such famous men of science, holding opposite points of view, should bitterly clash.

Inherently Pasteur was predisposed to consider yeasts as living organisms. He prepared pure cultures of these organisms which he studied with the aid of his beloved instrument, the microscope. Liebig, who firmly adhered to "puritanical" chemical methods, had no use for the microscope. In the fermentation of natural substances such as grapejuice and beer wort by yeast, Pasteur soon recognized the presence of impurities as well as other numerous complicating factors. His was not an easy task for, at that time, bacteria were not known and neither sterilization by heat nor pasteurization had been discovered. He prepared culture fluids containing sugars, inorganic phosphates and an ammonium salt as the only source of nitrogen. Thus the science of microbiology was born.

Pasteur's research on fermentation covered the span of more than two decades. It began shortly after he went to Lille, where he remained for three years, and was continued with even greater zeal after he was called to Paris, in 1857, to become the head of "Ecole Normale." His work and publications on this subject received much criticism from his opponents. However, he was not dismayed, but stoutly maintained that yeast is a living organism and, in so doing, incurred the lasting hostility of Liebig. Pasteur was not only a brilliant and methodical experimentalist but a great fighter who was able to confound his critics. In a recent lecture delivered by Alexander Fleming* we find the following quotation taken from Pasteur's work:

"I can bear witness to the existence of a large number of distinct yeasts setting up chemical transformations in accordance with their nature and constitution; but most frequently the nourishment suited to one allows others to develop. Hence arise most complicated phenomena, liable to constant variations. If one does succeed in separating one of these ferments and making it grow by itself, it produces the corresponding chemical change with remarkable precision and simplicity."

The question of spontaneous generation had been debated for centuries. After Pasteur had demonstrated that fermentation is caused by living organisms, he asked himself the all-important question: "Where did these living organisms or ferments originate?"

Pasteur's classical research on the origin of contamination, which unlocked the door to successful bacteriological research, can be summed up in his own words: "The dust suspended in the air is the only origin and the first and essential condition of life in infusions." His simple experiment, which consisted of introducing the fermentable fluid into a flask that had been drawn out in a narrow swan's-neck form, and sterilizing the contents of the flask by boiling, is one that is familiar to all. No fermentation took place in it, whereas fermentation did occur when the same fermentable fluid was placed in a flask the neck of which was not drawn out, so that after boiling and letting it stand in the laboratory the contents were exposed to the air. He concluded from this and other experiments that the dust-

* Brit. Med. J., *1*, 4502 (1947).

bearing germs in the air settled on the outside of the drawn-out neck of the flask and could not reach the sterile liquid.

Pasteur's first publication on the subject of alcoholic fermentation appeared in 1857. In 1860, with the application of sterile technic, he startled the world by his report that yeast can grow in a simple synthetic medium, the chemical constituents of which were known.

In recognition of his work he was given, by the French Government, a yearly pension of 20,000 francs for life.

On November 14, 1888, Pasteur's Institute was inaugurated by President Carnot. Pasteur was indeed overwhelmed, and in the speech which he delivered at the inauguration, he made a statement that should be engraved in our hearts, never to be forgotten:

"I would say that two contrary laws seem to be wrestling with each other nowadays: the one a law of blood and death, ever imagining new means of destruction and forcing nations to be constantly ready for the battlefield — the other a law of peace, work and health ever evolving new means of delivering man from the scourges which beset him. Which of these two laws will ultimately prevail God alone knows."

When someone once remarked to Pasteur how certain scientists were lucky in making accidental discoveries, Pasteur replied with a little twinkle in his eye: "Fortune favors the mind that is prepared."

In 1895, a few months before his seventy-third birthday, Pasteur's health failed, and on September 28 he died in peace.

Historical

Growth of Yeast in Media

In 1860 Pasteur [97] published his "Mémoires sur la Fermentation Alcoolique," in which he reported the results of his studies on the propagation of yeast in simple nutrient solutions containing mineral salts, sugars and ammonium tartrate. His medium consisted of 10 grams of sucrose (rock candy), 100 cc. of pure water, 0.1 gram of ammonium tartrate and 1 gram of yeast ash. Into this medium he introduced a yeast culture the size of a pinhead. He observed that a small culture permitted a slow onset of fermentation whereas a heavier culture, a more rapid one followed by evolution of gas bubbles, the criterion he employed for following the course of fermentation. Pasteur thus laid the foundation for microbiological research which during the past few years has assumed such an enormous magnitude that the biochemical literature is devoting considerable attention to its development. For those who are interested in this field of endeavor it is cogent to sketch a brief survey of the early literature.

In 1864, Duclaux [18] confirmed Pasteur's findings and declared that yeast could utilize the nitrogen of ammonium tartrate, ridiculing Millon's statement that Pasteur's yeast did not utilize the nitrogen in Pasteur's solution

but that the nitrogen evaporated into the air. Pasteur may have used an unnecessarily large amount of yeast for inoculation, but microbiology was not yet a science.

In 1871, following the termination of the Franco-German War, there was much bitter feeling between the two nations and Liebig, who supported an older theory of alcoholic fermentation, hotly contested Pasteur's findings and vigorously denied the possibility of obtaining either growth or fermentation in media of mineral-salt-sugar solutions. Thereupon Pasteur wrote a "Mémoire" in 1871 [98] in reply to Liebig's criticism, another one in 1872,[99] and in 1874 he published a confirmatory report entitled "Production de la Levure dans un Milieu Mineral Sucre." [100] In 1879, Nägeli [94] remarked that the weight of beer yeast in media containing sugar solution and ammonium tartrate could be increased twelvefold by aeration, but that the propagated yeast cells were rich in fat, poor in nitrogen and weak in their ability to effect fermentation.

In 1894, Beijerinck [6] observed that *Schizosaccharomyces octosporus* did not grow well in media containing ammonium salts and asparagine or in peptone alone. He asserted that the most effective source of nitrogen for yeast growth was that found in malt and grapes.

Discovery of "Bios"

At the turn of the century, a new light was shed on the question of yeast growth in synthetic media for, in 1901, Wildiers [139] reported his discovery of a growth factor in his publication, "Une Nouvelle Substance Indispensable au Developpement de la Levure." He termed this unknown factor "bios." Wildiers' medium contained 20 grams of sucrose, 5 grams of a salt mixture of magnesium sulfate, potassium chloride, ammonium chloride and disodium phosphate, and 1 gram of calcium carbonate dissolved in 200 grams of water. He employed fermentation flasks each containing 125 grams of well aerated medium with 10 grams of sugar, each inoculated with varying amounts of a culture *Saccharomyces cerevisiae* I Hansen from sterile beer wort, and kept at 28° C. The flasks were weighed every day for five days and the loss in weight was interpreted as due to evolution of carbon dioxide. He did not observe any loss in weight in the flask that received 2 drops of culture but the one that received 5 drops lost 5 to 5.5 grams. In a further investigation of this phase of fermentation, Wildiers observed that the increased fermentation caused by heavy seedings was not due to the effect of the increased number of yeast cells but to a certain unknown chemical substance which was provided by the filtrate of boiled yeast cells. He therefore concluded that Pasteur must have used large seedings of yeast in his experiments. Wildiers also reported that this unknown substance "bios" was water-soluble, insoluble in absolute alcohol but soluble in 80 per cent alcohol, stable on boiling for 30 minutes in 5 per cent sulfuric acid, dialyzable, and found in yeast, in Liebig's meat extract

and in commercial peptone but not present in yeast ash nor precipitated by lead acetate.

While Wildiers' report contained several inconsistencies and weaknesses, nevertheless it created considerable interest, and his theory of a growth factor for yeast was a lively controversial subject for many years. One cannot be harshly critical of Wildiers' concept of "bios" nor of the underlying principle that such a growth factor was indispensable for propagation and the proper functioning of microorganisms. Wildiers' report appeared at an early date. Scientists had not yet awakened. They were at the dawn of a new era that was to be replete with unbelievable scientific discoveries. Tryptophane had just been discovered but not its indispensability as an amino acid. Methionine and threonine were unknown The term "vitamin" had not as yet been coined. Epinephrine, insulin, thyroxine and other hormones were still in the ductless gland begging to be liberated. Tanner, in his review, "The 'Bios' Question," [133] stated: "Most of the investigators since 1901 have been concerned with establishing the correctness or disproving Wildiers' statement that an hypothetical substance to which he gave the name 'bios' was necessary for the normal growth of yeasts. It is interesting to note, however, that very few, especially those in America, have tried to use Wildiers' technic. Perhaps a better starting point would have been the use of these various factors such as medium, species of yeast, and other details of technic.

"One who has special training in the methods of microbiology also wonders whether the technic used in some of the experiments which have been reported, has been in accord with that used by trained bacteriologists and microbiologists. For instance, some authors relied on pasteurization, another on boiling for sterilizing their media. It is well known that numerous bacteria exist which form very resistant spores. One bacteriologist has reported an organism, the spore of which resisted boiling for 17 hours. Other illustrations could be mentioned to support this. It is obvious that the methods of sterilization should be rigorous and sufficient to insure sterility. A perusal of the descriptions of technic also causes one to question the sterility of the preparates even though sterile media may have been used. In some cases no attempt seems to have been made to determine whether bacteria were present. It is known that the mutual relationships of microorganisms are important. Some bacteria may inhibit the development of yeasts, others may favor it. Several statements appear in the publications on 'bios' to indicate that the growth of the yeast was influenced by contaminating bacteria."

Cogent and valuable information may be obtained through intelligent search of the early literature wherein, not infrequently, the solution of a problem may be hidden or which, through stimulation, may lead to the birth of new ideas. Research breeds research and ideas develop new ideas.

Even with our present knowledge of hormones, vitamins, indispensable

amino acids, enzymes, and essential mineral requirements, we do not have a clear concept of the exact role of a "growth factor." We also find that the term "growth stimulant" has been used to indicate a certain group of compounds, such as adenine, guanine, xanthine, thymine, etc., which do not belong to the above list of MUST substances.

With the discovery of vitamins it was debated whether or not "bios" was identical with the vitamin B found in yeast. In 1919 and 1920 Williams,[140, 141] who favored the idea that "bios" could be the antineuritic factor, suggested the use of yeast for the microbiological assay of the vitamin. However, the time was not yet ripe to perfect such a delicate test as our knowledge of the requirements of microorganisms was still obscure. As is common knowledge, what was once the water-soluble vitamin B blossomed into a complex group of important factors, many of which were found to be important accessory factors for animals, man, and even the despicable bacteria.

At about that time vitamins were in the early stages of development. Specific vitamin deficiencies were not clearcut since what was once considered one vitamin was subsequently shown to consist of more than one. They were concentrates of extracts of plants or tissues and the evaluation of their potencies depended on tedious and costly biological methods of assay which retarded progress in perfecting methods of purification, isolation, identification and synthesis.

Development of Synthetic Media

While the microbiologists were searching for ways and means to harness the inherent potentialities of microorganisms that had been primed, nursed and highly bred on the best "delicacies" that a scientific laboratory can offer, another group of investigators was working on the development of simple synthetic media.

The term "synthetic medium" is hereby defined as a culture medium containing ingredients of known chemical structure. Uschinsky [136] was one of the earliest investigators on record to make use of synthetic media containing ammonium lactate and asparagine as the only source of nitrogen for the growth of certain strains of *C. diphtheria* and the production of diphtheria toxin. He observed that older cultures grew better in his synthetic media than fresh cultures. In 1907, Hadley [50] repeated Uschinsky's experiments but was not able to confirm his findings except in two cases; he subsequently substituted glycine for asparagine and claimed good bacterial growth.

In 1919, Davis and Ferry [17] reported that the addition of cystine to a broth culture medium that is barely capable of maintaining the growth of the diphtheria bacillus permitted luxuriant growth and toxin production.

In 1921–1922, Braun and Cahn-Bronner [7] stated that 18 out of 22 strains could grow aerobically when ammonia was the sole source of nitrogen. However, it was observed that growth was frequently delayed for several

days or even weeks, but when once started the culture could readily be propagated in the same medium. The remaining 44 strains did not respond to the nitrogen of ammonia nor to that of a number of amino acids. These authors, however, stated that growth of these latter strains could be obtained in the presence of 0.5 per cent tryptophane. Later they expressed doubts as to the tryptophane requirements of these organisms. During the years 1920 to 1922, Mueller [90–92] demonstrated the presence of two unknown factors in protein hydrolysates necessary for the growth of streptococci. He developed a procedure for tracing these chemicals not unlike the microbiological methods employed years later for the identification of vitamins and growth factors. Von Groer's synthetic media [45] contained ammonium lactate, dipotassium hydrogen phosphate, sodium chloride and glycerol. The organism *C. diphtheria* did not grow well at first, but with subsequent culturing von Groer succeeded in getting it to adapt itself with the fabrication of a labile toxin. Several other bacteriologists have also attempted the propagation of this and other pathogenic organisms with little success. In some instances, instead of using simple known compounds as the sole source of nitrogen, some investigators introduced into their media such complex substances as peptones, sera, albumin, fibrin, etc., along with a haphazard number of amino acids. While such work is significant from an immunological and bacteriological point of view, the media employed cannot be classified as synthetic.

In 1927, Gibbs and Rettger [36] made an observation on the growth-promoting effect of cystine on this organism in 0.5 per cent concentration in a sugar-free veal infusion; however, when the amount of cystine in the medium was raised to 1 per cent there was an inhibitory effect. These authors also investigated tryptophane in their media and showed that it stimulated heavy growth when added in small amounts and in large amounts caused inhibition. In 1928, Jensen and Falk [68] reported that the addition of small amounts of cystine enhanced toxin production. Braun and Mündel[9] were of the opinion that cystine was an essential amino acid for the diphtheria bacillus. Wadsworth and Wheeler [138] reinvestigated Uschinsky's medium and showed that their *C. diphtheria* strain, Park 8, became attenuated after 25 to 30 successive transfers. In 1930, Maver [80] studied the nutrition requirements of several strains of *C. diphtheria* Park 8 with regard to growth and toxin production. She modified Braun and Hofmeier's [8] synthetic medium and reported that, of the ten amino acids she used, alanine, phenylalanine, valine and glycine were better growth stimulants to her organism than either leucine, histidine, tryptophane, glutamic acid, tyrosine or aspartic acid. She also observed that the addition of cystine to the modified synthetic medium enhanced toxin production.

In 1926, Gordon and M'Leod [40] classified the amino acids they studied with respect to their effect on the growth of certain bacteria into three groups: (a) Those indifferent toward growth, (b) those favoring growth,

and (c) those that are inhibitory. The results of these investigators may be objected to on the basis that fairly large amounts of amino acids were used and that peptone and not a simple synthetic medium was employed. Hosoya and Kishino [67] reported that they were able to grow *Cl. botulinum* on a gelatin medium which contained no tryptophane. Burrows [11-13] studied the requirements of *Cl. botulinum* and found that tryptophane and cystine possessed growth stimulating effect. He also investigated the growth of *Cl. botulinum* in synthetic media containing as the sole source of nitrogen purified amino acids and in others containing acid hydrolysates of casein or of gelatin.

The effect of amino acids on the growth of microorganisms may be due to three factors: (a) The availability of the nitrogen and carbon of the amino acids, (b) the inability of the organisms to synthesize a certain amino acid which is essential to its protein constituent, and (c) the influence of a certain group of amino acids on growth. In an investigation on infusoria (paramecium and ameba), Hammett [51] reported that sulfhydryl compounds stimulate the rate of cell division, but that the oxidation products of these compounds such as the sulfoxide, sulfonate and sulfinate retard growth.

In 1930, Yü,[147] working in Mueller's laboratory, isolated a strain of diphtheria bacillus which he showed to be a strong toxin producer and studied its growth requirements in an acid hydrolysate of casein to which were added tryptophane, Liebig's meat extract, and inorganic salts. In 1933, Mueller, Klise, Porter and Graybiel [93] worked on the fractionation of Liebig's meat extract, the acid hydrolysate of casein and the growth of Yü's strain of diphtheria bacillus. These authors concluded that tryptophane and cystine were essential for the growth of this microorganism along with two unidentified fractions, one of which was isolated from Dakin's butyl alcohol extraction method of amino acids and the other from Liebig's meat extract.

In 1933, Fildes, Gladstone and Knight [32] investigated the amino acid requirements of *B. typhosus*. They studied the effect of 14 amino acids and concluded that their microorganism could derive its nitrogen from a mixture of amino acids containing tryptophane, but ordinarily it would not grow without it. They added, however, that *B. typhosus* could be trained to grow with ammonia as the sole source of nitrogen.

The year 1933 marked the beginning of a decade of great activity in the field of microbiology, particularly in a search for better and more exacting knowledge of the nutritional requirements of microorganisms. There was greater emphasis on the use of simple compounds of known chemical structure and of growth factors in media. The controversial "bios" was again assuming prominence in a new attire. It was the opening of a new chapter in microbiology.

Growth Factors

Early Development

In a brilliant series of investigations emanating from Knight's laboratory, Bland-Sutton Institute, Middlesex Hospital, London,[32-34, 37-39, 71-75, 105] valuable reports were published on the bacterial requirements of amino acids and the indispensability of certain specific growth factors. One of Knight's outstanding achievements is the discovery, isolation and identification of nicotinic acid and nicotinamide as growth factors.

It may be pointed out that, while some investigators have heretofore been partially successful in growing certain pathogenic bacteria and producing their toxins in synthetic media, nevertheless on serial transfer the organisms either became attenuated or lost their ability to produce their toxins. It is possible that several investigators may have incorporated in their synthetic media all amino acids essential to their nutritional requirements but growth factors and other requirements were sadly lacking. Although Wildiers, after the turn of the century, had indicated the necessity of a growth factor for yeast, his observations and findings were ignored by many workers in this field. Whether or not his "bios" consisted of one or more growth factors was immaterial; likewise the chemical identity of "bios" was not as yet of great moment. The hub of the matter was whether or not microorganisms required one or more growth factors besides some mineral salts, energy-producing substances and nitrogenous compounds. The story of the development of vitamins is a shining example. Had we waited to isolate the pure vitamins in pure crystalline states and to determine their chemical structure, our knowledge might have been no greater now than it was some thirty years ago. Vitamin B, the water-soluble, antineuritic factor, was considered one substance. Many years elapsed before separation and identification of many of the sub B members of this group of vitamins were accomplished.

Bacteriologists recognized the importance of special media for the growth of microorganisms, particularly the pathogens. They realized that these were exacting bacteria whose habitat was animal tissues. In this connection, Mueller and his coworkers [93] aptly summed up the situation: "Without speculation as to the possible applications to bacteriological methods which might develop from a more exact knowledge of this sort, there is the probability that many facts of general biological importance would result. The various types of pathogenic bacteria under consideration, including some staphylococci, streptococci, and pneumococci, the diphtheria bacillus, the meningococcus and gonococcus, the influenza bacillus, as well as others less well studied, grow naturally within or upon the animal body, in the presence of an exceedingly complex mixture of substances. In the test tube, they flourish progressively better as the medium is enriched by more and more complex products, serum, whole

tissue, blood, etc. Repeated efforts to substitute the known chemical components of the animal body have either failed or been only partially successful. One is therefore forced to the conclusion that substances of still unknown composition, or of unrecognized importance are to be found in the body, a knowledge of which would be as important to the biochemist and physiologist as to the bacteriologist, if from perhaps different points of view.

"The view is generally held among bacteriologists that certain of the so-called 'enriching' substances, such as blood or serum, owe their influence to the presence of native proteins, and that in certain instances (gonococcus) even the species of animal from which the protein is derived may be important. There is also the possibility that tissue enzymes may be directly involved, or that certain colloidal or perhaps surface-tension conditions of the body fluids are difficult to duplicate in artificial mixtures. While such considerations indicate the complexity of the problems involved, there is abundant evidence that a number of comparatively simple, or at least relatively stable chemical constituents also play an important role. In most cases where an attempt has been made, chemically, to define the nature of growth requirements among these various organisms, the results have been sufficiently encouraging to warrant a continuation, while at the same time showing very clearly the practical difficulties. Fractions obtained in certain instances have been effective in such great dilution that the 'vitamine' conception has been definitely suggested, with all the uncertainties which have developed from many years' investigation of these substances in animal nutrition. Until the chemical nature of the vitamines, as well as that of the various substances involved in bacterial nutrition is much better understood than at present, it is obviously not possible to know definitely what the relation between them may be. *In any case, bacterial growth as a test is infinitely more practicable than animal feeding experiments, and, while obviously less satisfactory than chemical analysis, approaches it in rapidity.*"

Mueller and his coworkers were indeed correct in their predictions as we shall see later.

At about that time the author became interested in synthetic media and the growth of microorganisms. He and his coworkers [109] investigated the amino acid requirements of *E. coli*, the preparation of growth factors, and the effect of the latter on the growth of *E. coli* in the continuous flow system as devised by Cleary, Beard and Clifton.[16] The ability of the activating substance to maintain effect on the growth of *E. coli* over a long period of time was demonstrated. The "constant flow chamber" described by Cleary, Beard and Clifton provides for continual renewal of food from a reservoir into culture chamber and the simultaneous elimination of an equal amount of fluid from the chamber, thus holding to a minimum the accumulation of metabolites. This device was set up in duplicate, using

1.0 per cent peptone as medium, labeled A and B, and their populations allowed to reach a stable level. Activator to the amount of 10.0 cc. per liter was added to one reservoir and its effect watched by population counts. At intervals the position of the reservoirs was changed, "A" reservoir to "B" culture flask, or the reverse as the case might be. The rise and fall of the populations following the change demonstrated the accelerating effect on growth. Sahyun et al.[109] also investigated the utilization of the nitrogen of 19 amino acids by *E. coli* and noted considerable variation in the rate of utilization of the various individual amino acids. This was suggestive that this organism, under certain conditions, could be a useful tool for the microbiological assay of certain amino acids. Moreover, it was reported that the addition of cysteine and of tyrosine in small amounts stimulated and in larger amounts inhibited the growth of *E. coli*.

One of the peculiar phenomena in microbiology for which we have no satisfactory explanation is that bacterial dissociation may and often does occur under apparently optimum cultural conditions. In 1934, Manwaring [85] stated:

"There is apparently convincing evidence that hormones, chemical 'organizers' or integrating enzymes play an important role in stabilizing bacterial populations, and in initiating or inhibiting dissociations or transformation phenomena. The integrating factor thus far studied in greatest detail is the type-specific 'activator' of the pneumococcus.

"Under routine cultural conditions the pneumococcus breeds true as a capsulated diplococcus, whose type-specific diagnostic character is due to the presence of a highly specialized capsular polysaccharide. Some of these capsular sugars are of known chemical composition. Under certain slightly adverse cultural conditions, however, the capsulated pneumococcus dissociates into a non-capsulated variant, in which the most delicate immunochemical test fails to reveal a trace of its original type-specificity polysaccharide. This naked variant may breed true for innumerable test-tube generations.

"If, however, under certain serological conditions, a pure culture of this naked variant is enriched by the addition of an aqueous extract of alien-type pneumococci, the naked variant is 'activated' to a regeneration of its outer defensive capsule. The regenerated capsule, however, is not of the original type-specificity. The pneumococcus now manufactures a new capsular polysaccharide, apparently identical with that of the alien extract with which it was 'fertilized.' Thus hormonally hybridized to an alien type, the recapsulated pneumococcus breeds true for innumerable test-tube generations. It is as though albino crows stained with bluejay extract should transmute into a new and stable race of blue crows.

"Similar hormonal transformations are alleged for the tubercle bacillus, an unknown hormone, for example, capable of transmuting the conventional waxy tubercle bacillus into a stable, non-waxy variant. Of even greater

clinical interest, however, is the rapidly increasing evidence that hormonal transformations even take place between widely different bacterial species or genera. It is well confirmed, for example, that partial functional and chemical convergence takes place between certain pathogenic and non-pathogenic bacteria of the gastro-intestinal type if grown together in the same fluid medium. Even such widely different microorganisms as the streptococcus and the diphtheria bacillus demonstrably hybridize. Unfortunately, however, from the point of view of medical research, the resulting symbiotic or synergistic hybrids are rarely stable, reversion to type almost invariably taking place on separating the two associated species."

We are now in a better position to understand certain phases of bacterial growth as we have at last succeeded in propagating certain microorganisms in simple synthetic media. In investigations dealing with the nutritive requirements of different animals and of man we do not have to contend with sterile aqueous environment nor with pH control, temperature or buffered solutions. Our environmental problems are in a certain sense easier to control. Bacteria are more exacting and many of them are sensitive to minor alterations in their environment. The nutritive requirements of animals and of man are water, mineral salts, vitamins and proteins, fats and carbohydrates. With the possible exception of fats, all these nutrients are probably required by microorganisms.

It must be recognized that the bacteriologist has diligently worked out good culture media, a unique system of identification of organisms based not only on morphological characteristics but on metabolism, requirements of certain specific substances, and the liberation of identifiable by-products such as acids and alcohols, etc. With the development of synthetic media and a better knowledge of growth factors, microbiological methods of assay were assured of success. In this connection, it is noteworthy that a system for the assay of simple sugars by yeast cells had been instituted.

Microbiological Assay of Sugars

Chemical methods for the determination of certain carbohydrates seem at first sight to consist of simple, accurate procedures. They are performed routinely. However, they do not distinguish between true reducing sugars and non-sugar reducing substances such as are found in blood and in tissues. Also, some of the sugar methods do not show with certainty the nature of the sugar being determined. Carbohydrates of the nature of fructose, mannose, galactose, maltose, lactose, etc. give almost similar responses when treated with the alkaline copper sulfate or ferrocyanide reagents used for detecting and estimating dextrose. Thus no sooner were these methods developed than their shortcomings became known and they were showered with criticism.

From 1917 to 1923, several reports appeared relative to the identification of sugars by selective fermentation action of different organisms.[15, 69, 70]

Hiller, Linder and Van Slyke [62] made use of baker's yeast for the determination of fermentable sugar in blood. In 1927, Somogyi [127] noted that unwashed yeast retains reducing substances and advocated, for greater accuracy of fermentable blood sugar analysis, the washing of yeast cells until they are free of reducing substances.

In 1931, Harding and Grant [53] showed that baker's yeast can be acclimatized to ferment galactose as well as glucose. In 1933, Harding, Nicholson and Grant [55] found that *Saccharomyces marxicanus*, which ferments galactose, was useful as an analytical reagent for this sugar. In 1933, Harding and Nicholson [54] outlined a microbiological system of carbohydrate analysis embracing glucose, fructose-mannose, galactose, sucrose, maltose and lactose.

Microbiological Assay of Growth Factors

With the realization that most bacteria do require certain indispensable factors for growth, reproduction and the fulfillment of their other physiological functions, bacteriologists and chemists joined forces in an attempt to elucidate this problem. It was finally recognized that amino acids, sugars, and essential mineral salts were not sufficient for the growth of the exacting organism. In his review, Williams,[142] who had previously supported Wildiers' theory, pointed out the relationship of "bios" to the water-soluble vitamin and showed that under appropriate conditions pure vitamin B_1 has a stimulating effect on yeast growth. The second member of the B complex group considered essential to certain bacteria was discovered by Williams and was subsequently shown to be indispensable to all animals tested. It became known as pantothenic acid. These two discoveries were soon followed by another — pyridoxin, known as vitamin B_6, and this factor was shown to possess a strong stimulating power on yeast growth. Shortly thereafter vitamin H was isolated from yeast extract containing the "bios" growth factor mixture. The term "vitamin H" was shortlived, for on its synthesis it was termed biotin. The next member of this group noted to exert growth-stimulating effect on microorganisms was p-aminobenzoic acid. It was soon followed by the discovery of folic acid.

Knight,[72] who for many years has been investigating the amino acid requirements of microorganisms in synthetic media, discovered nicotinic acid and nicotine amide and showed that either compound is indispensable to the growth of *Staphylococcus aureus*. Riboflavin and choline have also been found to influence the growth of certain bacteria.

As more concrete evidence has been gathered by numerous workers, it has become apparent not only that these specific growth factors can be used to promote growth and physiologic function of microorganisms in suitable synthetic media, but that, through growth and or metabolism, some bacteria can be applied to trace very minute amounts of specific growth factors and vitamins.

In addition to the aforementioned vitamins, several other substances that enhance bacterial growth were discovered. Some of these have been chemically identified while others are as yet unknown. The chemically known compounds that possess growth-stimulating effect for certain bacteria are certain purines and pyrimidines. They are particularly important to several strains of group A hemolytic streptococci. Bacteriologists had already shown that animal sera must be added to the media for the successful propagation of this strain of bacteria. Wilson [143] found that xanthine, guanine, hypoxanthine, guanosine and adenosine could replace serum in the media. Pappenheimer and Hottle [96] reported that adenine was inactive although it stimulated growth of other similar organisms.

Table 1. Bacteriological Assay Methods [103]

Growth Factor	Test Organism	Principle of Measurement	Time of Test	Range of Standard per ml.
			hrs.	
p-Aminobenzoic acid	*Acetobacter suboxydans*	T	48	0-10 mγ
	Clostridium acetobutylicum	T	20-24	0-0.15 mγ
	Lactobacillus arabinosus	A	72	0-0.05 mγ
Biotin	*Clostridium butylicum*	T	72	0-0.1 mγ
	Lactobacillus arabinosus	A	72	0-0.25 mγ
	Lactobacillus casei	A	72	0-1 mγ
	Leuconostoc mesenteroides	T or A	72	0-1 mγ
	Rhizobium trifolii	T	72	Yeast extract
Choline	*Pneumococcus* Type III	T	12-24	0-6 γ
Coenzymes I and II	*Hemophilus influenzae*	Nitrite production	48	0-0.037 γ
	H. parainfluenzae	T	24-30	Yeast extract = 0-0.18 mg of fresh yeast
	H. parainfluenzae	T	40-42	2.5-20 mγ
	H. parainfluenzae	T	24-29	0-0.06 γ
Nicotinamide	*Shigella dysenteriae*	T	16-22	0-0.025 γ
Nicotinic acid	*Acetobacter suboxydans*	T	48	0.25-3.0 γ
	Bacillus proteus	T	30	0-0.1 γ
	Lactobacillus arabinosus	A	72	0-0.1 γ
	L. arabinosus	CO_2 liberation by acid formed	3	0-13.3 mγ
	L. casei	A	72	0-0.1 γ
	Leuconostoc mesenteroides	T or A	72	0-0.1 γ
	Dysentery bacillus (Shigella?)	A	4 days	0.001-0.010 γ

Table 1 (*Continued*)

Growth Factor	Test Organism	Principle of Measurement	Time of Test	Range of Standard per ml.
			hrs.	
Pantothenic acid	*Lactobacillus arabinosus*	A	72	0–0.02 γ
	L. casei	A	72	0–0.20 γ (Ca salt)
	Leuconostoc mesenteroides	T or A	72	0–10 mγ
	Proteus morganii	T, pH or bacterial nitrogen	24	0–1 mγ
(β-Alanine)	*Corynebacterium diphtheriae*	Bacterial nitrogen	70	0–1.5 γ
Pyridoxine * (pyridoxal, etc.)	*Lactobacillus casei*	A	72	0–0.1 γ
	Leuconostoc mesenteroides	T or A	72	0–0.25 γ
"Pseudopyridoxine"	*Streptococcus lactis R*	T	16	0–0.3 γ
Riboflavin (small amounts)	*Lactobacillus casei*	A	72	0–0.05 γ
	L. casei	A	72	0–20 mγ
	L. jugurt	A	72	0–0.1 γ
Thiamine	*Lactobacillus fermentum*	T	16–18	0–5 mγ
	Leuconostoc mesenteroides	T or A	72	0–3 mγ
	Propionibacterium pentosaceum	CO_2 evolution	4	0–0.25 γ
	Staphylococcus aureus	T	36	0–0.5 mγ
	Streptococcus salivarius	T	24	0–0.2 mγ
Norite eluate factor	*Lactobacillus casei*	A	48	0–300 γ solubilized liver fraction
Folic acid	*Streptococcus lactis* R	T	24	0–200 γ liver extract B
	Lactobacillus casei	A	72	0–0.003 γ folic acid concentrate
Vitamin B_c	*L. casei*	A	30–72	0–1 mγ
	S. lactis R	A	30–72	0–8 mγ
	S. lactis R	T	16	0–5 mγ

T = Turbidimetric measurements A = Acidimetric measurements

* Pyridoxine is inactive for *L. casei* and other lactic acid bacteria, whereas pyridoxal and pyridoxamine are very active.

Note: It has been reported that strepogenin may be an essential growth factor for *Streptococcus faecalis* [135] and *Lactobacillus casei*.[117]

Loring and Pierce [79] noted that pyrimidine glucosides have greater effectiveness on growth of certain neurospora than the free pyrimidines. Subsequently, Pierce and Loring [104] investigated the comparative effectiveness of free purines and purine glucosides and concluded that they were equally effective on molecular basis.

In 1920, Mueller [90] had shown that an enzymatic digest of casein contained an unknown factor that promoted the growth of streptococcus. In pursuing its isolation he had discovered methionine. In 1936, Sahyun et al.[109] investigated the effectiveness of an unknown growth factor from protein hydrolysate on the growth of *E. coli*. Recently Woolley [145] and Sprince and Woolley [129, 130] isolated an unknown substance from the enzymatic digest of casein and reported its stimulating effect on the growth of hemolytic streptococci. They called it "strepogenin." These authors are of the opinion that it is a peptide.

Other known and unknown growth factors have been reported and will eventually be discovered, as from all indications this field of research is still in its infancy. For detailed descriptions of the relation of vitamins and other growth factors to microorganisms, the reader is referred to the excellent reviews of Peterson,[102] Peterson and Peterson,[103] Koser and Saunders,[76] Snell,[124] and Williams.[142]

Need for a Microbiological System of Amino Acid Assay

The knowledge that had been gained through the years from investigations involving the growth of microorganisms in synthetic media finally led to the development of satisfactory, accurate microbiological methods of assay of growth factors and vitamins. In 1935, Schopfer [111] devised a bacterial method for the quantitative estimation of vitamin B_1, which was subsequently modified by several investigators.[10, 52, 88] In 1941, Snell and Strong [125] developed a method for riboflavin which involved the use of certain strains of lactic acid bacteria. Owing to its accuracy and simplicity, the method of Snell and Strong gained rapid acceptance and it was soon followed by other similar methods.

It so happened that these developments coincided with renewed interest in amino acids and proteins in nutrition. Chemical methods for the quantitative determination of amino acids either in the free state or in proteins have been too numerous — a probable indication of dissatisfaction among workers in this field as to their reliability. Investigators are aware of the many and formidable difficulties attending amino acid analysis. A protein hydrolysate is not as simple as it appears to be and, using Stein's words, "it is a witches' brew . . . a charm of powerful troubles." Progress in protein chemistry and a clearer understanding of protein nutrition cannot proceed at a rapid rate until we have an accurate and complete knowledge of the chemical composition of the protein molecule.

Many chemical procedures have been devised: (1) Fischer's esterification

system of analysis, (2) colorimetric methods, (3) methods employing precipitants believed to be specific for certain amino acids, (4) analytical methods based on solubility products, (5) isotope dilution methods, and (6) chromatographic and ion exchange methods. Although the simple and rapid methods have their place in many fields of research, there is that element of uncertainty as to accuracy and specificity. Also, methods of isolation and identification of amino acids have been cumbersome and wasteful. Some of the isolated, naturally-occurring amino acids that had been prepared in an allegedly high state of purity were frequently observed to contain impurities. In some instances the nature of impurity is negligible but in others, such as with the contamination of isoleucine with leucine or of leucine with methionine, impurities are serious in nature and cause considerable confusion. In view of this and of the high cost of optically active amino acids, the chemist directed his attention to synthesis. Considerable strides were made and most of the essential as well as a large number of the non-essential amino acids became available at comparatively reasonable cost. However, unlike the naturally-occurring constituents of proteins (glycine excepted), the products of syntheses are optically inactive; and, since practical methods for the resolution of the racemic amino acids have not as yet been satisfactorily worked out, racemization added to the complexity of the problem and necessitated additional chemical and biological research. It became of considerable importance to determine the availability of the optically inactive amino acids by the animal system and their fate in metabolism. In this connection, one must bear in mind that, whatever conclusion is arrived at from findings obtained in one species of animals, such conclusion does not necessarily apply to other species. The literature is replete with differences in various species requirements. Vitamin research and studies on dispensability and indispensability of certain amino acids have clearly indicated how cautious we should be in this respect.

Again, as biological methods of assay of vitamins have proved to be tedious and time-consuming, biological methods for the evaluation of proteins and amino acids are at least equally tedious and time-consuming. However, in the absence of shorter and more reliable methods, biological methods are a necessity.

We have learned that the nutritive value of a protein is dependent not only on the presence of all essential amino acids among its constituents but on the amounts and availability to the body of these substances. Despite the formidable difficulties encountered by the protein chemist in assigning exact values for amino acids to proteins, valuable information has been obtained. However, some of the data published have been incomplete and some have been misleading. In some instances, the published erroneous data were inevitable and errors could be attributed to lack of proper procedures of isolation and imperfect analytical methods. In other instances, the protein chemist has no one to blame but himself. The literature includes

reports on the amino acid content of proteins based on the assumption that the latter contain exactly 16 per cent nitrogen. Needless to say, all proteins do not contain this exact amount. For a thorough evaluation of the constituents of proteins it is of paramount importance to use purified samples, particularly if one wishes to devote months and perhaps years to a study of their composition. Many of the proteins that are being analyzed consist of denatured commercial preparations. From a nutritional point of view, it is of course desirable to know their nitrogen as well as their amino acid content. However, it is not necessary to complicate the picture by recalculations of results on an arbitrary figure of 16 per cent nitrogen. These proteins are fed to animals and to man either as the sole source of nitrogen in diets or in combination with other proteins. The following quotation from a discussion by Vickery [137] on "Contribution of the Analytical Chemist" is pertinent to this subject:

"Kossel pointed out that the decomposition products mentioned represented experimentally a large number of different proteins, but that, nevertheless, most proteins yielded many of them. Accordingly, the protein molecule, doubtless, must be extraordinarily complicated, and the development of a structure formula promised to be an assignment of the greatest difficulty, even if preparations of demonstrated purity could be secured — in itself an extremely difficult task. Kossel also emphasized the wide differences that had been found in the analytical composition of proteins with respect to amino acids, especially in the cases of arginine and of glutamic acid. His own work upon the protamines had revealed the existence of protein-like substances, some of which yielded more than 80 per cent of arginine, while such plant proteins as zein yielded as little as 1.8 per cent. Thus, the proteins comprised a group of substances of the widest possible variety in composition. In the final paragraph, he summed up the situation as follows: 'It has been customary to think of protein as a substance of definite fixed properties, a sort of "ideal protein," much as Goethe thought of an "*Urpflanze*" or "ideal plant." Those protein substances that did not correspond to this ideal have been supposed to be defective and have been placed in a lower group, such as the albuminoids. This point of view can no longer be maintained. It is a necessity in present day science to regard each organic substance as a member of an evolving series, a necessity that has its most striking expression in the direction taken by phylogenetic and ontogenetic research. We must not, therefore, consider one complicated protein molecule to be representative of all, but must seek to find a system of proteins which, progressing from the simplest to the most complex, reveals to us the innermost character of these many sided substances.'"

Another important consideration in the pursuit of analytical methods is the critical examination of the proper methods of hydrolysis of proteins and the preclusion of the occurrence of amino acid decomposition particularly during the conversion stage of peptides to amino acids. It is well known

that amino acids are more susceptible to decomposition when combined in peptide linkage than when they are in the free state.

The microbiologist has now entered a fertile field of intricate research. With the aid of his newly acquired technic he has evolved ingenious methods for the microquantitative assay of amino acids. There is hope that he will avoid many of the pitfalls of the analytical chemist and will always endeavor to provide accurate descriptions of his methods as well as careful specifications for media, the care of his test organisms, and critical measurements of results. The microbiologist deals with very minute amounts of amino acids and a slight error in making up standards for growth or in the preparation of standard solutions for the measurement of acids produced by the test organism or in measurement of growth by a turbidimetric apparatus, etc., will yield unreliable results. As a rule, both chemical and microbiological methods of assay have their limitations and shortcomings. These must not be overlooked by the investigator, otherwise the values obtained will create greater confusion.

In their detailed review of bacteriological assays for growth factors, Peterson and Peterson [103] state:

"Theoretically an assay method should be possible for any compound that is required by a microörganism, but in practice some microörganisms are more satisfactory than others. Some of the necessary conditions that should be fulfilled are as follows:

"1. The medium should contain all constituents that are necessary for optimum development and activity of the microörganism other than the factor to be determined. Such a medium is indicated by the production of a low turbidity or acidity in the absence of the factor and optimum growth and formation of products in its presence. With an excess of the factor the development of the microörganism should be equal to that in a natural medium of comparable sugar and nitrogen content.

"2. The response of the microörganism (as measured by cell growth, products, or other index) to increasing quantities of the factor should be regular and preferably proportional. Equal response to all forms of the compound on a molar basis is desirable. . . ."

"3. In the assay of natural materials the responses at different levels should give the same value when this is calculated per gram or other unit of the material. Irregular values are indicative of another factor or factors in the material which may be stimulatory or inhibitory at different levels.

"4. Repeated assays of the same material should check, *e.g.*, within 5%. A suitable sample repeatedly analyzed serves as a reference material and should be included in every large series of analyses as a useful check on the assay as a whole.

"5. The sample should contain no inhibitory or toxic substances. If such substances are present, the results will probably be variable, as mentioned under 3. Such a substance may be present in the original material

or be produced by the treatment of the material, *e.g.*, decomposition products or excess salts.

"6. A standard inoculum should be used. To start with a sturdy and stable microörganism is essential. The stock culture should be carried on a medium that maintains the organism in a stable condition, and the inoculum should be developed in the same way each time. Obviously such points as age and size of inoculum are of great importance.

"7. The microörganism should preferably be non-pathogenic. If the method is to be widely used and consequently by technicians, a pathogen is dangerous, and necessary precautions reduce the number of assays that can be performed in a given time.

"8. The method should be rapid. In research work, the time required for an assay should be 1 to 3 days, and for control work in industry 5 to 20 hours.

"Even if these requirements are met to a reasonable degree, there still is no substitute for skill and experience on the part of the analyst. Perfection in the method should not be expected. Even after more than half a century of use and scores of modifications the Kjeldahl method for total nitrogen is still not perfect. If used with skill and judgment, microbiological assays can be valuable and useful tools."

The development of microbiological methods is indeed a challenge to the protein chemist. Instead of deterring him from pursuing his search for better chemical methods it should stimulate him to increase his efforts. Better and simpler chemical methods for amino acid analysis are sorely needed. Better preparations of pure, optically active amino acids and pure proteins must be available not only for standard purposes but to permit a better understanding of the chemistry of the protein molecule. The domain of the protein chemist has been invaded, on one side, by the organic chemist who is synthesizing amino acids and, on the other, by the microbiologist who is quantitatively evaluating the amino acid content of proteins.

Microbiological Methods of Assay of Amino Acids

Schweigert and Snell [116] summarize the advantages and disadvantages of microbiological methods for the assay of amino acids as follows:

1. They are highly specific and sensitive and a four-fold or even greater reduction in scale is possible.

2. They eliminate many of the laborious separations previously necessary in protein analyses.

3. They are applicable in a number of instances where no other method suitable for routine application has been developed and several estimations are little more trouble than a single one.

Their present disadvantages include the following:

1. Unfamiliarity to most chemists of an analytical technique which

requires control of a multitude of factors, some of which are imperfectly understood.

2. Danger of the lack of activity of the unnatural isomers may cause these to be overlooked. For some applications, of course, their specificity is advantageous.

3. Possibility that unrecognized factors may combine to alter the specificity of the test organism in a given instance. This risk is always present.

For the quantitative estimation of amino acids by microorganisms, important consideration must be given to: (1) Test organisms, (2) media, and (3) measurements of growth and criteria for establishing accuracy of results.

Test Organisms

Assay of amino acids by these methods involves the use of certain *homofermentative* or *heterofermentative* groups of bacteria as test organisms.

The *homofermentative* group includes those bacteria (such as *Lactobacillus casei, L. arabinosus, L. pentosus, L. delbrückii, Streptococcus faecalis*, etc.) that are capable of almost quantitatively converting glucose to lactic acid (1 M of glucose $\longrightarrow$ 2 M lactic acid).

The *heterofermentative* group includes those bacteria (such as *Lactobacillus fermentum* and *Leuconostoc mesenteroides*) that are capable of converting glucose to lactic acid as well as to other degradation products of fermentation as ethyl alcohol and carbon dioxide.[101]

Although many organisms have been employed for microbiological assay of amino acids, only a limited number have been carefully studied and recommended by workers in this field. One reason is that different strains of the same species may not give the same response or may differ from one another as to their specific nutritional requirements. Thus, whenever microbiological estimates are reported, it has become customary to mention the strain number as well as the species; e.g., *Lactobacillus arabinosus* 17–5, *Lactobacillus casei* Be-1, *Lactobacillus fermenti* 36, *Lactobacillus delbrückii* 5, etc. As in analytical chemistry one has to be careful about the purity and standardization of reagents, so in microbiology one has to exercise considerable care in the selection of test organisms and tend to their proper culture. Microbiologists may use different technics for culturing organisms but, whatever procedure is adopted, the test organism must be free from other bacteria, and its exact nutritional requirements must be provided.

Recently Dunn et al.[30] studied the response of 23 lactic acid bacteria grown in a basal medium of arbitrary composition and reported that the number of amino acids essential for these microorganisms ranged from two (glutamic acid and valine) for *Leuconostoc mesenteroides* 8293 to 15 (exclusive of serine, alanine, proline, hydroxyproline, norleucine and norvaline) for *Lactobacillus brevis* 8257. As thorough searches are made, the number of bacteria that can be used as test organisms for amino acids may become

unlimited. It is also possible that future research in this field will enable us to assay with similar accuracy whole proteins present in a complex mixture. In 1934, Sahyun and Beard [108] demonstrated that insulin could be purified without loss of potency by a certain strain of *E. coli* (a non-proteolytic organism) whereas its potency and its nature were destroyed by proteolytic organisms such as *Proteus vulgaris*.

In culturing the organism some prefer a stab culture in an agar medium [125] containing 1.5 per cent agar, 1.0 per cent yeast extract and 1.0 per cent glucose, or a basal liquid medium.[25] Others have recommended rich media.[95] Transfers may be made once a month or more frequently, that is, once a week. Cultures are incubated for 24 or 48 hours until good visible growth is attained and kept at a temperature of about 4–18° between transfers.

For quantitative microbiological assay it is mandatory that (1) test organisms specifically require for growth one or more amino acids, and (2) growth media be free from the amino acid to be determined but contain all other indispensable nutrients for adequate growth.

Media

Special media for test organisms must be prepared and tested before quantitative microbiological assay of amino acids is attempted. Most media (reported in the literature) for these tests are similar to one another and consist of: (1) Amino acids and, at times, other nitrogenous substances, (2) growth factors including vitamins, (3) mineral salts, (4) buffers, and (5) suitable carbohydrates for energy requirements.

Amino Acids. For assay purposes, media may preferably contain all known naturally occurring amino acids but must lack the specific amino acid to be determined. The amounts of each amino acid should be such as to yield optimum growth for turbidimetric or acidimetric measurements. In some instances, tryptophane-free acid hydrolysates of casein have been used for the assay of tryptophane [11] and glutamic acid-free hydrolysates for glutamic acid (glutamic acid being removed by a chemical procedure).[78] By acid hydrolysis of oxidized casein [131] the hydrolysate becomes suitable for methionine assay.[106]

In Table 2 the amino acids that have been used for test organisms by different investigators are listed. For the preparation of a suitable acid hydrolysate of proteins, a period of 10-hour autoclaving at 15 pounds pressure of one gram of protein in 10 per cent HCl has been recommended.[132] The autoclaved sample is neutralized with sodium hydroxide and diluted to the desired volume. The removal of acid is not necessary. Acid hydrolysis by autoclaving of samples for 30 hours or longer has given evidence of racemization or slight destruction of certain amino acids.[132] Prolonged acid hydrolysis may cause intercondensation or interaction of certain amino acids at elevated temperatures such as between tryptophane and cystine,

Table 2. Composition of Typical Test Media Used by Seven Different Groups of Workers [116]

(The numbers at the heads of the columns refer to the publication in which the medium is described)

Constituent per 10 ml. Medium	Amount of Constituent in Medium for Three Test Organisms						
	L. arabinosus		*S. faecalis*		*Leuconostoc mesenteroides*		
	85	153	33[c]	43	31	46	2
Carbohydrate, buffer and salts, mg.							
Glucose	100	200	100	200	200	100	200
Sodium acetate	60	200	60	—	120	60	120
Sodium citrate	—	—	—	250	—	—	—
KH_2PO_4	10	5	5	—	5	80	5
K_2HPO_4	10	5	5	50	5	80	5
$MgSO_4 \cdot 7\,H_2O$	2	2	2	2	2	2	2
$FeSO_4 \cdot 7\,H_2O$	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$MnSO_4 - 7\,H_2O$	0.1	0.1	0.1	0.1	0.1	0.1	0.1
NaCl	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$(NH_4)_2SO_4$	30	—	—	—	60[c]	30	60[c]
Vitamins and growth factors, μg.							
Thiamine hydrochloride	1.0	2.0	2.0	2.0	10	5.0	10
Riboflavin	1.0	4.0	2.0	2.0	20	2.0	20
Nicotinic acid	1.0	4.0	2.0	6.0	20	2.0	20
Biotin	0.002	0.001	0.002	0.002	0.05	0.01	0.05
Vitamin B_6	3.0[a]	2.0[a]	4.0[b]	12.0[a]	16[a]	100[a]	16[a]
p-Aminobenzoic acid	3.0	1.0	0.1	—	0.001	3.0	0.001
Folic acid [d]	0.03	—	0.007	0.02	0.01	0.03	0.01
Calcium pantothenate	1.0	2.0	2.0	1.0	10	2.0	10
Adenine sulphate	100	100	100	100	120	100	120
Guanine hydrochloride	100	100	100	100	120	100	120
Xanthine	100	—	—	100	—	100	—
Uracil	100	100	100	100	120	100	120
Choline chloride	25	—	—	—	—	25	—
Inositol	25	—	—	—	—	25	—
Amino acids, mg.							
Aspartic acid	4.0	4.0	1.0	4.0	4.0	4.0	4.0
Glutamic acid	10.0	4.0	1.0	4.0	1.5	10.0	5.0
Arginine monohydrochloride	1.0	0.5	2.0	1.0	0.8	3.0	2.5
Histidine monohydrochloride	1.0	0.5	2.0	0.5	0.2	1.0	1.0
Lysine monohydrochloride	1.0	2.0	1.0	2.0	0.8	1.0	1.25
Alanine	1.0	1.0	1.0	0.5	10.0	1.0	5.0
Cystine	1.0	1.0	2.0	2.0	1.2	1.0	1.0
Glycine	1.0	—	2.0	0.2	1.0	1.0	1.0
Hydroxyproline	1.0		2.0		1.0	1.0	—
Isoleucine	1.0	1.0	1.0	1.0	0.75	1.0	1.0
Leucine	1.0	1.0	1.0	2.0	0.75	1.0	1.0
Methionine	1.0	0.5	1.0	0.5	0.20	1.0	1.0
Phenylalanine	1.0	0.5	1.0	0.5	0.30	1.0	0.5
Proline	1.0	—	2.0	—	0.25	1.0	1.0
Serine	1.0	—	1.0	0.5	0.40	1.0	0.5
Threonine	1.0	1.0	1.0	1.0	2.25	1.0	2.5
Tryptophane	1.0	0.4	2.0	1.0	0.10	1.0	0.5
Tyrosine	1.0	0.4	2.0	0.8	0.30	1.0	1.0
Valine	1.0	1.0	1.0	1.0	0.75	1.0	1.0
Norleucine	1.0	—	1.0	—	0.5	1.0	0.5
Norvaline	1.0	—	—	—	0.5	1.0	0.5

[a] Pyridoxine hydrochloride was used. [b] Pyridoxamine dihydrochloride was used.

[c] Ammonium chloride was used in place of ammonium sulphate.

[d] Expressed in terms of pure folic acid, recalculated where necessary on the assumption that pure folic acid has a potency of 137,000.

[e] All comparisons of amino acid concentrations are on the basis of the amount of the *l*-isomer added. If *dl*-amino acids were used, the amount added was twice that indicated. This procedure seems justified, since with very rare exceptions the unnatural isomers of the amino acids are not available for growth. [f] Asparagine was used in place of aspartic acid.

serine and threonine.* On the other hand, short periods of hydrolysis of proteins by acids will not sever all peptide linkages. It has also been pointed out that certain amino acids in weak concentrations promote growth and in high concentrations inhibit growth of certain bacteria.[68, 109] Further investigation of inhibitory amounts of amino acids to organisms is suggested.

Several investigators have studied with varying success the microbiological assay of the products of alkaline hydrolysis of proteins by different concentrations of sodium hydroxide and barium hydroxide and at different temperatures.[41, 132, 144] Some of the causative factors for the inconsistencies of microbiological results encountered in the use of alkaline hydrolysates of proteins could be (1) incomplete racemization of the optically active amino acid during the course of digestion, (2) difference in time intervals for complete racemization of different amino acids, and (3) decomposition of certain amino acids.[107] †

Horn and Jones[61] and subsequently Greenhut et al[43] investigated the use of enzymatic digests of proteins of certain foods and reported satisfactory results for tryptophane assay methods. However, since enzymes do not effect complete digestion of proteins to amino acids, the indiscriminate use of enzymes is bound to introduce into the enzymatic hydrolysates unknown factors as the result of enzyme breakdown as well as of associated nitrogenous impurities. Furthermore, there is evidence[55, 77, 78, 81, 116, 132] to show that inconsistent estimates were obtained for amino acids when synthetic peptides were used for microbiological methods.

Table 3. Microbiological Assay Ranges for the Various Amino Acids ‡

Amino Acids	Ranges of Amounts per 10 cc. of Basal Media	
	Minimum gamma	*Maximum* gamma
Alanine	40	500
Arginine	10	80
Aspartic Acid	50	500
Cystine	10	120
Glutamic Acid	50	500
Glycine	10	100
Histidine	5	50
Hydroxyproline	—	—
Isoleucine	10	100
Leucine	10	100
Lysine	10	200
Methionine	10	100
Phenylalanine	10	150
Proline	10	80
Serine	50	250
Threonine	50	500
Tryptophane	2	25
Tyrosine	5	80
Valine	10	100

‡ Values in this table are taken from publications by different workers in this field.

* Sahyun, M., Unpublished data.

† Kuiken, K. A., and Lyman, C. M., *Federation Proc.*, **6**, 269 (1947).

Growth Factors and Vitamins. The importance of inclusion of these substances in synthetic media has already been discussed. For the microbiological assay of amino acids, amounts of these substances used in various media are as a rule in excess of the minimum requirements of test organisms. (See Table 2.)

Mineral Salts. The various mineral salts used in media for microbiological assays have been patterned after Speakman's [128] with certain modifications, such as the addition of manganese which has been shown to be required by some lactic acid bacteria.[89, 126, 145]

Potassium salts have also been incorporated as they have also been demonstrated to enhance growth of *Streptococcus faecalis*.[80]

Buffers. For optimum growth of the lactic acid bacteria and lactic acid production, sodium acetate and sodium citrates have been substituted for phosphate buffers, partly because of the buffer weakness of the latter in overcoming the acidity of lactic acid as formed by the test organism. This is indeed important for lactic acid formation can well overcome the effect of a phosphate buffer and alter the pH of the medium to a point where it will become detrimental to the test organism. Assuming that 0.1 N lactic acid * is formed during the course of fermentation, then according to the law of mass action,

$$H = \sqrt{K(x)}$$

where H is hydrogen in concentration, K dissociation constant, and x concentration of acid. For 0.1 N lactic acid, $K = 1.38 \times 10^{-4}$. Thus,

$$H = \sqrt{1.38 \times 10^{-4} \times 0.1} = 10^{-2.57}$$
$$pH = -\log_{10} H, \text{ or } pH = 2.57$$

At pH 2.57 organisms are either inactivated or destroyed.

Energy Requirements. For microbiological assay of amino acids, the incorporation of carbohydrates for energy requirements of test organisms is not only necessary but their nature and the amount used must be known and properly controlled. For methods depending on lactic acid production and its quantitative measurement as an index of the indispensable amino acid on test, glucose in amounts of 100 mg. per 1 cc. of medium has been found to be almost completely converted to lactic acid, provided all other nutritional requirements have been met. Recently, Camien, Dunn and Salle [14] made a thorough investigation of the response of different organisms to various sugars. They studied 24 strains of lactic acid bacteria and showed that of the 22 carbohydrates and carbohydrate derivatives, glucose, in most instances, yielded the maximum amount of lactic acid.

For heterofermentative organisms such as *Leuconostoc mesenteroides*, the amount of glucose in the medium is usually increased (for acidimetric measurement) since this group of bacteria produce substances other than

* 100 mg. of glucose will permit the production of approximately 100 mg. of lactic acid, about 11 cc. of 0.1 N acid.[116]

lactic acid from glucose. Obviously, with an increase in the amounts of sugar, a corresponding increase in the amounts of buffer is advisable to care for excess acid production.

Table 4. Microbiological Assay of Amino Acids

Amino Acid	Test Organism	References
Alanine	*Streptococcus faecalis*	30, 43, 83
	Leuconostoc mesenteroides	30
	Lactobacillus arabinosus	30, 83
	Lactobacillus casei	30
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30
Arginine	*Streptococcus faecalis*	1, 4, 30, 43, 46, 63, 83, 110, 132
	Leuconostoc mesenteroides	4, 30
	Lactobacillus arabinosus	30, 83, 87
	Lactobacillus casei	4, 5, 30, 60, 61, 87
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30
Asparagine	*Streptococcus faecalis*	30, 43
	Leuconostoc mesenteroides	30
	Lactobacillus arabinosus	30
	Lactobacillus casei	30
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30
Aspartic Acid	*Streptococcus faecalis*	83
	Leuconostoc mesenteroides	2, 48, 110
	Lactobacillus arabinosus	83
	Lactobacillus delbruckii	106, 131
Cysteine	*Streptococcus faecalis*	30
	Leuconostoc mesenteroides	30
	Lactobacillus arabinosus	30
	Lactobacillus casei	30
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30
Cystine	*Streptococcus faecalis*	48, 83
	Leuconostoc mesenteroides	110
	Lactobacillus arabinosus	1, 83
Glutamic Acid	*Streptococcus faecalis*	3, 22, 30, 43, 83, 110
	Leuconostoc mesenteroides	3, 22, 30
	Lactobacillus arabinosus	3, 5, 19, 30, 49, 61, 78, 81, 83, 110
	Lactobacillus casei	3, 5, 30
	Lactobacillus fermenti	20, 30
	Lactobacillus delbruckii	30
Glutamine	*Lactobacillus arabinosus*	49
Glycine	*Streptococcus faecalis*	30, 43
	Leuconostoc mesenteroides	30, 119
	Lactobacillus arabinosus	30
	Lactobacillus casei	30
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30

Table 4 (*Continued*)

Amino Acid	Test Organism	References
Histidine	*Streptococcus faecalis*	4, 5, 30, 43, 83, 110, 132
	Leuconostoc mesenteroides	1, 4, 24, 25, 28, 30, 31, 46, 60, 61, 63, 120
	Lactobacillus arabinosus	30, 83
	Lactobacillus casei	30
	Lactobacillus fermenti	20, 28, 30
	Lactobacillus delbruckii	30
Isoleucine	*Streptococcus faecalis*	4, 30, 43, 63, 83, 132
	Leuconostoc mesenteroides	4, 30
	Lactobacillus arabinosus	1, 4, 5, 30, 57, 59–61, 83, 110
	Lactobacillus casei	4, 5
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30
Leucine	*Streptococcus faecalis*	4, 30, 43, 63, 83, 132
	Leuconostoc mesenteroides	4, 30
	Lactobacillus arabinosus	1, 4, 5, 30, 35, 57–61, 83, 110, 113, 120
	Lactobacillus casei	4, 5
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30
Lysine	*Streptococcus faecalis*	4, 5, 30, 43, 46, 83, 132
	Leuconostoc mesenteroides	1, 2, 23, 30, 31, 46, 60, 61, 63, 66, 110, 113
	Lactobacillus arabinosus	30, 83
	Lactobacillus casei	30
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30
Methionine	*Streptococcus faecalis*	4, 30, 43, 82–84, 106, 110, 132
	Leuconostoc mesenteroides	1, 2, 4, 21, 30, 31, 63, 82, 84, 106
	Lactobacillus arabinosus	4, 21, 30, 65, 106, 110
	Lactobacillus casei	30
	Lactobacillus fermenti	20, 21, 30
	Lactobacillus delbruckii	30
Phenylalanine	*Streptococcus faecalis*	30, 43, 83
	Leuconostoc mesenteroides	1, 2, 27, 30, 31, 63
	Lactobacillus arabinosus	30, 83, 110, 120
	Lactobacillus casei	4, 5, 27, 30, 43, 60, 61
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30, 132
Proline	*Streptococcus faecalis*	30
	Leuconostoc mesenteroides	2, 30, 110
	Lactobacillus arabinosus	30
	Lactobacillus casei	30
	Lactobacillus fermenti	30
	Lactobacillus delbruckii	30
Serine	*Streptococcus faecalis*	4, 30, 43, 83
	Leuconostoc mesenteroides	30, 110
	Lactobacillus arabinosus	30, 83
	Lactobacillus casei	30
	Lactobacillus fermenti	30
	Lactobacillus delbrückii	30, 151

Table 4 (*Continued*)

Amino Acid	Test Organism	References
Threonine	*Streptococcus faecalis*	1, 4, 5, 30, 43, 63, 83, 110, 132
	Leuconostoc mesenteroides	30
	Lactobacillus arabinosus	4, 5, 30, 59, 60, 61, 83
	Lactobacillus casei	30
	Lactobacillus fermenti	20, 29, 30
	Lactobacillus delbrückii	30
Tryptophane	*Streptococcus faecalis*	4, 23, 26, 30, 41-43, 83, 110, 118, 120, 132
	Leuconostoc mesenteroides	30
	Lactobacillus arabinosus	1, 4, 30, 42, 44, 56, 60, 63, 83, 84, 110, 112, 114, 115, 120, 144, 146
	Lactobacillus casei	4, 30
	Lactobacillus fermenti	30
	Lactobacillus delbrückii	30
Tyrosine	*Streptococcus faecalis*	4, 30, 43, 83
	Leuconostoc mesenteroides	30, 31, 63, 110
	Lactobacillus arabinosus	30, 83, 110
	Lactobacillus casei	4, 5, 30, 60, 61, 84
	Lactobacillus fermenti	30
	Lactobacillus delbrückii	30, 47
Valine	*Streptococcus faecalis*	4, 30, 43, 46, 63, 83, 132
	Leuconostoc mesenteroides	4, 30
	Lactobacillus arabinosus	1, 4, 5, 30, 35, 57, 59, 60, 61, 83, 87, 110, 113, 120
	Lactobacillus casei	4, 5, 30, 57
	Lactobacillus fermenti	30
	Lactobacillus delbrückii	30

Preparation of Media

As a rule, growth media are prepared in twice the final concentration of various ingredients (excepting the one amino acid to be tested) and adjusted to the proper pH suitable for optimum growth of the test organism. 5 cc. of the medium deficient in a single amino acid is introduced into test tubes and various concentrations of the chosen standard are added to one set and of the unknown sample to another. Concentrations of standards and unknowns must be in amounts calculated to produce from almost zero to maximum growth. Contents of all tubes are diluted with distilled water to the same volume (preferably to 10 cc.). They are then sterilized by autoclaving, cooled and inoculated with a suspension of the test organism obtained from an actively grown culture. The tubes are next incubated at a constant temperature suitable for the test organism and for a definite time recommended to yield optimum growth and/or lactic acid.

Measurements of Results

For turbidimetric measurements of bacterial growth, photoelectric colorimeters are generally used. It is essential that cells of the culture be well

shaken and time allowed for air bubbles to break. Readings are recorded, plotted and checked against a standard reference curve that had been constructed or as galvanometer readings against the known amounts of the amino acid added.

If titration measurements are desired, the acidity of a sample is determined by titration with a standard alkali.

According to Snell [121, 122] the criteria for testing the accuracy of a microbiological method is comprised of agreement of (1) values calculated from different assay levels and (2) results obtained with those of other methods that are known to yield accurate data. As in chemical methods, repeated assays by the same microbiological procedure must yield consistent results.

Table 5. Amino Acid Content of Proteins as Determined by Microbiological Methods by Different Workers

Amino Acids	Casein	β-Lactoglobulin	Egg Albumin	Fibrin	Silk Fibrin	Lactalbumin	Gelatin
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Arginine	3.7 (3)	2.9 (3)	5.75 (2)	7.2	1.10	3.2	8.65 (2)
Histidine	2.7 (3)	1.55 (2)	2.30	2.2	0.40	1.8	1.55 (2)
Isoleucine	5.9 (4)	8.4 / 7.0	7.00	5.9	1.15	6.0	1.55 (2)
Leucine	9.5 (5)	15.45 (2)	9.2	6.9	0.90	10.8	3.25 (2)
Lysine	7.8 (3)	11.25 (2)	6.6	8.5	0.70	8.4	4.10 / 5.8
Methionine	2.9	3.2 / 2.5	1.25	2.6	0.15	2.6	0.8 (2)
Phenylalanine	5.1 (3)	3.5 / 4.3	7.9	4.4	1.50	3.7	2.25 (2)
Threonine	4.0 (3)	5.9 / 4.6	3.6	6.4	1.40	5.4 (2)	1.95 (2)
Tryptophane	1.19					2.06	
Valine	6.5 (5)	5.7 (3)	6.9	5.5	3.35	5.9	2.6 (4)
Glutamic Acid	22.2 (4)	18.7	14.3		2.03		10.5 (2)
Aspartic Acid	7.03	11.5	9.0				
Tyrosine	5.0 (2)						
Glycine	1.95 (5)				44.9 (5)		
Alanine	1.90						
Cystine	0.30						
Proline	8.70						
Hydroxyproline	0.20						
Serine	5.0						
Ammonia	1.6						

The percentages for each amino acid are averages of several values found by different workers and the figures in parentheses represent the number of determinations. Minima and maxima are given for estimates that are not in close agreement. Single figures represent findings of one investigator.

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Appendix

List of U. S. Patents Issued on Amino Acids and Related Organic Compounds

Compiled by

Dean Laurence

Patent Attorney, St. Johns, Michigan

U. S. Patent No.	Title and Patentee	Date Issued
544 933	Citrate of Ethenylethylenamidin and Process of Obtaining Same *Albrecht Schmidt*	1895 Aug 20
662 754	Cyanmethyl-Anthranilic Acid and Process of Making Same *Fritz Bender*	1900 Nov 27
699 581	Indigo-Diacetic Acid and Process of Making Same *Paul Seidel*	1902 May 6
716 242	Disazo Dye and Process of Making Same *Paul Julius*	1902 Dec 16
952 006	Preparation of Esters of Oxyamino Acids *Ernest Fourneau*	1910 Mar 15
1 035 591	Nutritive and Flavoring Substance and Process of Making Same *Kikunae Ikeda* *Saburosuke Suzuki*	1912 Aug 13
1 344 673	Manufacture of Urea and of Intermediate Products *Carl Bosch*	1920 Jun 29
1 440 269	Pharmaceutical Compound *Rudolf Berendes* *W. Grutteſien*	1922 Dec 26
1 506 728	Method of Manufacturing Nitrotartaric Acid *Arthur Lachman*	1924 Aug 26
1 602 958	Flavoring Compound and Method of Making Same *Pao Nien Woo*	1926 Oct 12
1 634 221	Manufacture of Betaine Hydrochloride, etc. *D. K. Tressler*	1927 Jun 28
1 634 222	Process of Preparing Betaine Hydrochloride *Donald K. Tressler*	1927 Jun 28
1 637 661	Production of Organic Compounds *Karl Friedrich Schmidt*	1927 Aug 2
1 780 860	Betainethiocyanate *Rudolf Berendes* *Ludwig Schutz*	1930 Nov 4
1 890 590	Manufacture of Glutamic Acid Compounds *Fong Yih Sen*	1932 Dec 13
1 909 757	Process for Producing Commercial Ammonium Carbonate *Thomas Coxon*	1933 May 16

U. S. Patent No.	Title and Patentee	Date Issued
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1 938 651	Vulcanization of Rubber *Ira Williams*	1933 Dec 12
1 942 610	Process for Production of Water Soluble Metal Carbamates *Robert Burns MacMullin*	1934 Jan 9
1 948 002	Production of Carbamates *Alwin Mittasch* *Paul Chall*	1934 Feb 20
1 964 723	Production of Alkali Metal Carbamates *George Lewis Cunningham* *Robert Burns MacMullin* *Wesley King McCready*	1934 Jul 3
1 967 400	Production of Guanidino Fatty Acids *Siegfried Fischl*	1934 Jul 24
1 969 516	Production of Alkali Metal Carbamates *Robert Burns MacMullin*	1934 Aug 7
1 973 574	Process for Manufacturing and Recovering Glutamic Acid *A. E. Marshall*	1934 Sep 11
1 973 860	Amides of N-Beta-Hydroxyalkyl Amino Carboxylic Acids and Process of Preparing Same *Heinrich Ulrich* *Karl Saurwein*	1934 Sep 18
1 974 554	Process for the Production of Amino Acids from Substances Containing Keratin *Ernst Ziegler*	1934 Sep 25
1 976 997	Method of Separating Optically Active Glutamic Acid without Racemization *Seizo Kanao*	1934 Oct 16
1 983 041	Production of Carbamates of the Alkali-Forming Metals *Robert Burns MacMullin* *Wesley King McCready*	1934 Dec 4
1 990 769	Amino Acids *H. M. Barnett*	1935 Feb 12
1 992 462	Manufacture of Flavoring Materials *H. M. Barnett*	1935 Feb 26
1 992 804	Process of Producing Glutamic Acid Compounds *E. Bartow* *R. L. Albrook*	1935 Feb 26
1 998 485	Process for Obtaining Organic Nitrogen Bases *James R. Bailey*	1935 Apr 23
2 002 656	Process for the Production of Calcium Carbamate *Nikodem Caro* *Albert Rudolph Frank* *Hans Heinrich Franck*	1935 May 28
2 002 681	Process for the Production of Potassium Carbamate *Carl Theodor Thorssell* *August Kristensson*	1935 May 28

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2 004 523	Aminocarboxylic Acids and Salts Thereof *Reinhold Fick*	1935 Jun 11
2 009 868	Method of Preparing Leucine *H. M. Barnett*	1935 Jul 30
2 014 512	Production of Carbamates and Conversion Product of the Same *Alfred Stock*	1935 Sep 17
2 017 537	Production of Hydroxy-Alkylamino Carboxylic Acid Compound *Ulrich Hoffman* *Bernhard Jacobi*	1935 Oct 15
2 023 890	Method of Eliminating Knocking in Internal Combustion Engines *Leo B. Kimball*	1935 Dec 10
2 041 265	Acylated Polypeptides *Ludwig Orthner* *Georg Meyer*	1936 May 19
2 049 480	Oxidation Products of Cystine and Related Compounds and Process for Making the Same *Gerrit Toennies*	1936 Aug 4
2 049 576	Method of Preparing Meat Sauce *Otto Ungnade*	1936 Aug 4
2 050 491	Method of Preparing Sodium Glutamate *George Kumagai*	1936 Aug 11
2 067 013	Process for Producing the Disodium Salt of Imidodicarboxylic Acid *Robert B. MacMullin*	1937 Jan 5
2 071 253	Linear Condensation Polymers *Wallace H. Carothers*	1937 Feb 16
2 071 282	Method of Separating Amino-Acids Readily Soluble in Water and Ammonium Sulphate *Wilhelm Gluud* *Walter Klempt*	1937 Feb 16
2 071 327	Process of Racemizing Amino Acids *R. S. Bley*	1937 Feb 23
2 078 592	Oxidation Products of Cystine and Related Compounds and Process for Making the Same *G. Toennies* *T. F. Lavine*	1937 Apr 27
2 081 528	Wetting Agents for the Use in Mercerizing Lyes *K. Brodersen*	1937 May 25
2 085 784	Process of Purifying Aminated Compounds *R. R. Bottoms*	1937 Jul 6
2 090 068	2,4-Dioxo-3,3-Dialkyl-Tetrahydropyridines and Process for Manufacture of Same *Ernst Preiswerk* *Otto Schnider*	1937 Aug 17

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2 098 923	Process of Preparing Aminoacetic Acid *E. T. Mertz*	1937 Nov 9
2 109 929	Preparation of Amino Acids *G. W. Rigby*	1938 Mar 1
2 112 210	Process of Manufacture of Sulphur Containing Protein Compounds *Oskar Huppert*	1938 Mar 22
2 117 207	Amino Acids *L. Orthner*	1938 May 10
2 126 113	Cellulose Derivative Composition *Ralph Jacobson*	1938 Aug 9
2 129 264	Nitrogen-Containing Organic Compounds *Frederick Baxter Downing* *Frank Willard Johnson*	1938 Sep 6
2 130 505	Polyamino Carboxylic Acids and Process of Making Same *F. Munz*	1938 Sep 20
2 143 388	Manufacture of Hydroxyalkylated Nitrogen Bases *Paul Schlack*	1939 Feb 10
2 143 490	Manufacture of Condensation Products of Totally Hydrolized Protein Material and Products Thereof *Georg Meyer*	1939 Jan 10
2 146 873	Plastic Masses from Organic Colloids *Gustav Wilmanns*	1939 Feb 14
2 160 880	Lubricant *Clarence M. Loane* *Bernard H. Shoemaker*	1939 Jun 6
2 163 594	Process for the Production of Amino Acids *W. H. Engels* *G. A. Stein*	1939 Jun 27
2 164 781	Amino-Carboxylic Acids and a Process of Preparing Them *Carl Platz* *J. Rosenbach*	1939 Jul 4
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2 174 239	Preparing Acetyl Acetic Acid Derivatives *Anthony H. Gleason*	1939 Sep 26
2 176 785	Process of Glutamic Acid Production *G. Braun*	1939 Oct 17
2 178 210	Isolation of Leucine and Tyrosine from Corn Gluten *A. M. Mark*	1939 Oct 31
2 178 510	Removal of Chlorides from Solutions of Amino Acids *L. Gerber*	1939 Oct 31
2 180 636	Amino Acids Product and Method of Manufacture *K. S. Kemmerer*	1939 Nov 21
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2 183 856	Condensation Products of Hydrolized Protein Material and Their Manufacture *Georg Meyer*	1939 Dec 19
2 186 162	Process of Glutamic Acid Recovery from Solutions *G. Braun*	1940 Jan 9
2 191 738	High-Molecular Polycarboxylic Acid Amides and Their Production *G. Balle*	1940 Feb 27
2 191 978	Quaternary Nitrogen Compounds and Process of Preparing Them *Gerhard Balle* *Karl Horst*	1940 Feb 27
2 194 082	Method of Reacting Carbon Dioxide and Ammonia *Robert B. Booth*	1940 Mar 19
2 194 302	Separation of Amino Acids from Protein *L. Gerber*	1940 Mar 19
2 195 974	Process of Producing New Aminocarboxylic Acids *Walter Reppe* *Hanns Ufer*	1940 Apr 2
2 200 220	N-Substituted Aspartic Acids and Their Functional Derivatives and Process of Producing Them *Walter Reppe* *Hanns Ufer*	1940 May 7
2 203 009	Manufacture of N-Tertiary Amino Acyclic Carboxylic Acids and Their Salts *William Stansfield Calcott* *Louis Spiegler* *John Marlin Tinker*	1940 Jun 4
2 206 249	Agents of Capillary Action from Alkyl-Amino-Carboxylic Acids with Tertiary or Quaternary Bound Nitrogen *Karl Daimler* *Carl Platz* *Fritz Bucking*	1940 Jul 2
2 208 941	Hydroxy Mercury Compound and Method for the Production Thereof *Erno Geiger* *Laszlo Vargha* *Laszlo Richter*	1940 Jul 23
2 209 299	Glutathione Compounds and a Method of Making the Same *Walter Schoeller* *Hans Goebel*	1940 Jul 23
2 212 783	Cysteine Sulphinic Acid and Method of the Preparation Thereof *Theodore F. Lavine*	1940 Aug 27
2 212 847	Process for the Recovery and Reutilization of Ammonia and Carbon Dioxide in Urea Synthesis *Frank Porter*	1940 Aug 27

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2 215 367	Condensation Products of Acid Amide-like Constitution and Process of Preparing Them *Gerhard Balle* *Heinz Schild*	1940 Sep 17
2 217 846	Condensation Products of Betaine-like Constitution and a Process of Preparing Them *Ludwig Orthner* *Gerhard Balle* *Johann Rosenback* *Kurt Bonstedt*	1940 Oct 15
2 222 993	Process of Recovering Amino Acids *Gerrit Toennies*	1940 Nov 26
2 225 155	Preparation of Amino Compounds *Nicholas D. Cheronis*	1940 Dec 17
2 225 960	Condensation Products and a Process of Preparing Them *Ludwig Orthner* *Gerhard Balle* *Karl Horst*	1940 Dec 24
2 229 744	Diazoamino Compounds and Method of Making *Jean G. Kern*	1941 Jan 28
2 234 680	Process of Preparing Pantothenic Acid *Marjorie B. Moore*	1941 Mar 11
2 236 921	Process for the Manufacture of Therapeutically Valuable Compounds of Keratin Degradation Products *Karl Schollkopf*	1941 Apr 1
2 239 617	Preparation of Iminodiacetic and Aminotriacetic Acids *John C. Moore*	1941 Apr 22
2 239 706	Nitrogenous Compounds *Albert K. Epstein* *Morris Katzman*	1941 Apr 29
2 241 927	Process of Preparing Amino Acids *Melville Sahyun*	1941 May 13
2 243 437	Sulphonic Acid Amides and a Process of Preparing Them *Ludwig Orthner* *Gerhard Balle* *Karl Horst* *Heinz Schild*	1941 May 27
2 245 610	Medicinal Composition *Charles William Schaffer* *Reinhard Beutner*	1941 Jun 17
2 250 468	Product of Mild Oxidation of Long-Chain Hydrocarbons and Process of Producing It *Frank O. Cockerille*	1941 Jul 29
2 250 553	Medicinal Preparation *Simon L. Ruskin*	1941 Jul 29
2 251 892	Capillary Active Compounds and Process of Preparing Them *Ludwig Orthner* *Carl Platz* *Hans Keller* *Heinz Sonke*	1941 Aug 5

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2 261 002	Organic Nitrogen Compounds *David Moore Ritter*	1941 Oct 28
2 266 448	Process and Products for Deliming Limed Pelts *Otto Trosken* *Walter Pense*	1941 Dec 16
2 266 747	Water Soluble Substituted Amino-Methylene Mercapto Acids and the Process for Their Synthesis *Max Engelmann* *Emeric Havas* *Morris S. Kharasch*	1941 Dec 23
2 267 971	Process of Producing Glutamic Acid *Geza Braun*	1941 Dec 30
2 271 872	Production of Growth Promoting Substances *Herschel K. Mitchell*	1942 Feb 3
2 277 016	Preparation of Organic Compounds *Herbert H. Guest*	1942 Mar 17
2 279 908	Protein Digestion *G. B. Ayres* *J. G. Niedercorn*	1942 Apr 14
2 279 909	Protein Digestion *G. B. Ayres* *J. G. Niedercorn*	1942 Apr 14
2 279 910	Protein Digestion *G. B. Ayres* *J. G. Niedercorn*	1942 Apr 14
2 279 911	Protein Digestion *G. B. Ayres* *J. G. Niedercorn*	1942 Apr 14
2 281 612	Iodine Compound and Method for Producing the Same *Paul John Witte*	1942 May 5
2 284 086	Detergent Composition *Walter P. Ericks*	1942 May 26
2 301 829	Process for the Manufacture of the Lower Aliphatic Esters of Pantothenic Acid *Andre Studer*	1942 Nov 10
2 301 969	Monoamides of Polycarboxylic Acids *Walter Pinkernelle*	1942 Nov 17
2 306 646	Preparation of Monosodium Glutamate *Paul R. Shildneck*	1942 Dec 29
2 313 504	Sulphydryl Compound Obtained from Flour *Arnold K. Balls* *Walter S. Hale*	1943 Mar 9
2 313 573	Capillary Active Compounds and Process of Preparing Them *Ludwig Orthner* *Carl Platz* *Hans Keller* *Heinz Sonke*	1943 Mar 9
2 315 679	Process for the Preparation of the Cyanhydrin of Form-isobutyraldol *Kurt Warnat*	1943 Apr 6

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2 322 783	Preparation of Amides *Morris Katzman* *Albert K. Epstein*	1943 Jun 29
2 327 119	Process of Making Amino Acids *Elmore Louis Martin*	1943 Aug 17
2 328 021	Derivatives of Amines *Morris Katzman* *Albert K. Epstein*	1943 Aug 31
2 328 940	Preparation of Amino Acids *Witty Lysle Alderson, Jr.* *Paul Rolland Austin*	1943 Sep 7
2 331 677	Methylol Beta, Beta'-Iminodipropionic Acid *Roy S. Hanslick*	1943 Oct 12
2 334 163	Preparation of Beta-Alanine *Philip M. Kirk*	1943 Nov 16
2 334 986	Method of Hydrolyzing Protein Materials *Frederick C. Bersworth*	1943 Nov 23
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2 335 605	Preparation of Beta-Alanine *Joseph H. Paden* *Philip M. Kirk*	1943 Nov 30
2 335 653	Preparation of Beta-Alanine *Russell T. Dean*	1943 Nov 30
2 335 997	Production of Beta-Alanine and Beta-Alanates from Acrylonitrile *Gustaf Harry Carlson* *Charles Neil Hotchkiss*	1943 Dec 7
2 336 067	Preparation of Beta-Alanine *Gustaf H. Carlson*	1943 Dec 7
2 337 576	Benzylthiouronium Salt of Pantothenic Acid and Process of Making the Same *Eric Thomas Stiller*	1943 Dec 28
2 341 610	Process for the Manufacturing of Dextro-Pantothenic Acid *Andre Grussner*	1944 Feb 15
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